THE BIOCHEMICAL JOURNAL

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The Precise Determination of Haemoglobin in Blood by Abridged Photoelectric Spectrophotometry

By J. W. NICHOLAS
Silver End, Witham, Essex

(*Received* 10 *February* 1951)

Many workers (e.g. Karr & Clark, 1941; Macfarlane, 1945; Macfarlane, King, Wootton & Gilchrist, 1948) have drawn attention to the inaccuracy of most methods for the determination of haemoglobin in routine laboratory practice. McCarthy & Van Slyke (1939) have shown that there is a diurnal variation in the haemoglobin content of the blood of normal men amounting to a range of 7 % on the Haldane scale. If it is desired to follow closely the progress of an anaemia it is therefore essential that the already large uncertainty should not be increased still further by the use of a method only accurate to 10 % or worse.

Precise photoelectric methods of haemoglobin determination have been described by Szigeti (1940) and Bell, Chambers & Waddell (1945) among others. These workers use an incandescent light source, with approximately monochromatic filters. Their results, therefore, though highly reproducible, require the calibration of the individual instrument and filters against a blood solution whose concentration has been determined by other means. Absolute methods in general use depend on chemical determination of the iron content (e.g. Delory, 1943), or on manometric determination of the gas capacity (e.g. Van Slyke & Neill, 1924), and both these techniques are too time-consuming for frequent use. Spectrophotometry could also yield absolute values, provided an agreed spectrophotometric standard existed. The British Standards Institution (1942) has published the absorption curve of carboxyhaemoglobin used in calculating the tristimulus colour values by which the British Standard is defined; this curve is intended to be used as a whole, and therefore constitutes another tedious method. Because of the sharpness of the absorption bands of most blood pigments, spectrophotometric constants determined at single wavelengths are markedly influenced by small errors in wavelength setting, or differences in slit width. For this reason Butterfield (1912) suggested the use of the mercury arc with a spectrophotometer. Heilmeyer (1933) quotes Butterfield as having found an absorption ratio at 546 mμ. for oxyhaemoglobin corresponding to a specific extinction ($c = 1$ g./ml.) of 800.

Nicholas & Pollak (1950) have described filters which give sharp isolation of the mercury lines and therefore bring true spectrophotometric measurements within the realm of routine absorptiometry. It is the purpose of this paper to try to establish the spectrophotometric or abridged spectrophotometric* determination of haemoglobin as a method as fundamental as the iron content or gas capacity, and far more rapid.

A variety of haemoglobin derivatives have been used in colorimetric estimations. King *et al.* (1948) found oxyhaemoglobin itself to be one of the two most reliable of the six they examined. It has obvious advantages in convenience and in avoiding the introduction of errors by additional manipulation, and has therefore been chosen for the present method.

While this paper was in the press Donaldson, Sisson, King, Wootton & Macfarlane (1951) published spectrophotometric curves of haemoglobin derivatives, and deduced by interpolation between the 540 and 550 mμ. extinctions a value for oxyhaemoglobin corresponding to $E^{1\,\text{g./ml.}}_{546\,\text{m}\mu} = 810$.

METHODS

For the determination of the specific extinction of HbO_2 at 546 mμ., and for the investigation of the turbidity correction and the stability of HbO_2 the photoelectric absorptiometer described by Nicholas (1951) was used. The green line of the mercury arc (546 mμ.) and a band in the orange-red (designated '607 mμ.', but consisting largely of background spectrum, and therefore not strictly monochromatic) were isolated by the filter combinations of Nicholas & Pollak (1951). For experiments in routine determinations a model H 602 Spekker absorptiometer with mercury vapour lamp (made by Adam Hilger Ltd.) was also used, with the precautions detailed later. Oxalated human venous blood was diluted with 0·007 N-NH_4OH, which Szigeti has shown to favour the formation of methaemoglobin less than the more usually recommended higher concentrations. Carboxyhaemoglobin where required was prepared by shaking the diluted blood with excess of pure CO prepared from A.R. formic acid and H_2SO_4. The solution cell used for the determination of the specific extinction was specially selected and measured by the makers (The Tintometer Ltd.). A blank on

* This term is used here to denote the measurements of true densities at exact wavelengths by a shortened procedure. Its use as by some American authors to denote any absorptiometric procedure is considered incorrect.

water was measured in the same cell and subtracted. Comparison with the British Standard was made by the National Physical Laboratory on their comparator.

Choice and use of absorptiometer. An instrument is required equipped with a mercury vapour lamp and capable of having its density scale checked over the entire range used. The H 602 Spekker is the most commonly available instrument fulfilling these requirements, but the newer H 676 instrument is preferable. If facilities for its construction are at hand the Nicholas (1951) absorptiometer is especially suitable. Precautions necessary in using the Spekker for precision work are discussed at length by Pollak & Nicholas (1951). In particular it should be noted that the H 602 Spekker has the monochromatic filters in approximately the plane of the calibrated variable aperture, and the H 676 model has them in an optically equivalent position. Density gradients or other imperfections in the filter will therefore affect the calibration of the instrument (see example given by Nicholas, 1951). This means that if the instrument is not modified the same individual filter which was used during calibration must always be used for readings, and must always be inserted the same way up. Heat affects the transmission of filters as well as damaging them, so it is advisable to modify the H 602 Spekker as recommended by Hadley (1945) to bring them into a cool, constant and accessible position on the right-hand side of the drum. It is even better, if possible, to modify the instrument more drastically so that the filters are immediately adjacent to the right-hand photocell (the position of, and variations in, the left-hand filter are unimportant). If this is done the calibration not only becomes independent of variations in individual filters but also becomes the same for all wavelengths. It is essential, of course, that any modification made in the photocell housing should not allow the entry of stray light, which in this position would be especially detrimental to accuracy. The elimination of the left-hand water cell is recommended to avoid the zero-drift which results from air-bubble formation there; Nicholas & Pollak (1951) have found that this cell serves no useful purpose. The possibility of lost motion exists in the drum of the H 602 Spekker (but not the H 676), and the balance point should, therefore, always be approached in the direction of closing the drum aperture, where movement is positive. For convenience it is preferable to adopt a modification of Vaughan's (1941) 'water-to-water' setting and set the instrument 'air-to-air'. To ensure reproducibility the setting on the H 602 model should be at a drum reading not greater than 0·800. A lens should be used for both setting and reading; the latter should be by interpolation to 0·001.

Calibration of density scale. The Spekker scale, although bearing an approximately linear relationship with density, cannot be considered as a density scale. It should therefore be recalibrated by the following simple and rapid method. The procedure is described more fully by Pollak & Nicholas (1951), who also describe the process of cutting a new scale. In the following abridged description it is assumed that all that is required is the construction of a correction graph, or preferably, table. Using the 546 mμ. line the Spekker is set air-to-air 0·800, and a filter of density d (approximately 0·07), conveniently made by binding together two pieces of plain glass, is placed in a suitable holder in the cell carrier and a reading obtained for it. Leaving the drum at the reading so reached the galvanometer is restored to zero by the zero adjustment and a second reading of the filter taken. By this means drum readings corresponding to d, $2d$, $3d$, ..., are found. Finally the drum is reset to 0·800 and a plane-parallel glass neutral filter of known density D (in the region of 0·5) calibrated, for example, at the National Physical Laboratory is read. By linear interpolation D is expressed in terms of d, and thus the true value of d is found. A calibration graph can then be drawn, which will probably be nearly linear.

Filters. Owing to the sharpness of the β-absorption band of oxyhaemoglobin stray light either in the form of background radiation adjacent to the line or in the form of near infrared will cause large errors. Filters used therefore require to be chosen with care (Nicholas & Pollak, 1951). The combination Wratten 16 + Wratten 74 + Ilford 804 is recommended to isolate the 546 mμ. line for the actual determination. If it is desired to apply the turbidity correction (see p. 3) the band in the orange-red can be isolated by Wratten 26 + Ilford 804, but this requires a more sensitive galvanometer than the Cambridge Spot usually used with the Spekker; a much wider band not requiring a sensitive galvanometer could be isolated by Wratten 25 + Ilford 801. It is convenient to cement the individual gelatin films in the above combinations into compound filters. Light should traverse them in the order in which the components are listed. Manufacture of Wratten 16 and Wratten 26 has recently been discontinued in England, although stocks should last some time yet (Kodak Ltd., personal communication). Two thicknesses of Ilford 111 can be substituted for Wratten 16, and two thicknesses of Wratten 25 for Wratten 26 (Nicholas & Pollak, 1951).

Preparation and examination of blood. Oxalated, heparinized, or fresh blood may be used. A dilution of 1 in 250 in 0·007 N-NH_4OH gives a suitable density. If large numbers of examinations are being made a dilution of 1 + 200 will be found more convenient, the blood being measured in a 0·05 ml. capillary pipette and the diluent in a 10 ml. automatic pipette. The extinction of the solution is measured at 546 mμ. in a 10 mm. cell. The extinctions given in Table 2 will be found convenient in calculating results. For measurements of the greatest precision the dilute blood should be filtered (e.g. through Whatman no. 541 paper), but this is normally unnecessary. It should be remembered that tolerances on the nominal size of both pipettes and cells are relatively large (see p. 4); checking is therefore recommended.

RESULTS

Reproducibility

Ten replicate 1 in 250 dilutions of blood were prepared. The oxalated blood was mixed before sampling by gently inverting the bottle once a second for 60 sec. The same Ostwald-Folin pipette calibrated to contain 0·2 ml. and the same 50 ml. volumetric flask were used throughout. Densities were read on the author's absorptiometer on the day of dilution, and on the Spekker set air-to-air 0·5 on the following day. The absorptiometer gave a mean value of 0·3853, with a range of 0·385–0·387, and a coefficient of variation of 0·175 %. The Spekker gave a mean of 0·3855, range 0·385–0·387, coefficient of variation 0·184 %. It should be noted that this coefficient of variation takes account of all random errors of sampling from a bulk specimen of blood, pipetting, diluting and reading, but not of errors in the calibration of glassware.

Determination of specific extinction of oxyhaemoglobin

Blood was diluted 1 in 100 and filtered through Whatman no. 541 paper. Portions were saturated with carbon monoxide and submitted to the National Physical Laboratory (N.P.L.) for standardization. Further portions of the original ungassed solution were diluted 1 + 1 with 0·007 N-ammonia and their densities measured at 546 mμ. in a depth of 10·000 ± 0·005 mm. As a precaution against errors in diluting the same 10 ml. pipette was used for measuring both blood solution and diluent, and the dilution was performed in quadruplicate. Table 1 gives the

Table 1. *Determination of $E_{546\,m\mu}$ of oxyhaemoglobin corresponding to* 100 % *on British Standard Scale*

(Each mean represents three readings on each of four dilutions. The maximum deviation from the mean was 0·001, which occurred five times in all thirty-six readings.)

Exp. no.	% B.S.I. (N.P.L. result)	Mean extinction (1 in 200 dilution)	Extinction of 100 % B.S.I.	Mean
206	92·7	0·552	0·595	
207	106·9	0·635	0·595	0·5963
327	87·6	0·525	0·599	

results. The mean extinction of a 1 in 100 dilution of blood of 100 % haemoglobin on the British Standards Institution (B.S.I.) scale is therefore 1·192$_6$, with a possible error (see later) of ± 0·59 %. Taking 100 % B.S.I. as being 148 g. haemoglobin/l. (King *et al.* 1947) the specific extinction $E^{1\,g./ml.}_{546\,m\mu}$ becomes 806. This value assumes a factor for the iron content of haemoglobin of 0·334 %, based on the results of Hüfner (1894). If this factor is eliminated as possibly unreliable, and the haemoglobin molecule is assumed to contain four atoms of iron the molecular extinction $\epsilon_{546\,m\mu}$ is 53,910.

Table 2. *Extinctions of oxyhaemoglobin solutions at 546 mμ. for routine determinations*

Concentration of original blood	Dilution: 1 in 250	Dilution: 1 + 200
100 % B.S.I.	0·477	0·593
100 g./l.	0·322	0·401
1 mM	0·216	0·269

Stability of oxalated blood

In view of the possibility of a choice arising between doing a haemoglobin estimation immediately by a less accurate method and sending it elsewhere to be done accurately after one or more days' delay the effect of delay was investigated. Ten oxalated bloods which had been taken at intervals over a period of 12 days were diluted afresh and read 13 days after taking the first sample. The same glassware was used throughout. A comparison between the immediate and delayed readings is given in Table 3, which shows that in no case does the difference exceed 0·002 (approximately 0·5 % of reading).

Table 3. *Stability of oxalated blood*

Exp. no.	Age of specimen (days)	Original $E_{546\,m\mu}$.	Delayed $E_{546\,m\mu}$.	Difference
102*a*	13	0·385	0·383	−0·002
101*a*	12	0·402	0·402	0·000
102*b*	11	0·402	0·400	−0·002
101*b*	10	0·408	0·408	0·000
102*c*	9	0·387	0·386	−0·001
102*d*	6	0·363	0·365	+0·002
101*c*	5	0·363	0·365	+0·002
102*e*	4	0·389	0·389	0·000
101*d*	3	0·386	0·386	0·000
101*e*	2	0·386	0·385	−0·001

Effect of turbidity

It has sometimes been adduced against photoelectric haemoglobinometry that slight turbidities due to lipaemia may cause falsely high results. Such turbidities will be visible to the eye, and can be allowed for by making an extra reading at about 607 mμ. If the ratio $E_{546\,m\mu}/E_{607\,m\mu}$ is R, and the added turbidity is assumed to have equal densities at the two wavelengths, it can be calculated that the true extinction at 546 mμ. is

$$(\text{measured } E_{546\,m\mu} - \text{measured } E_{607\,m\mu}) \times R/(R-1).$$

To test this equation in use replicate dilutions of the same oxalated blood were made in which were incorporated varying amounts of milk, producing in some cases a turbidity in excess of anything likely to be found in practice. Corrected and uncorrected readings are given in Table 4. The value of $R/(R-1)$

Table 4. *Use of turbidity correction*

Exp. no.	$E_{546\,m\mu}$.	$E_{607\,m\mu}$.	$E_{546\,m\mu}$. corrected	Difference from expectation
91*a* (control)	0·389	0·025	0·388	−0·001
92*a*	0·449	0·084	0·388	−0·001
92*b*	0·515	0·155	0·383	−0·006
92*c*	0·571	0·209	0·386	−0·003
92*d*	0·416	0·048	0·392	+0·003
92*e*	0·407	0·041	0·390	+0·001
	Mean			−0·001
	Standard deviation			0·0031
	Coefficient of variation			0·81 %

used was 1·063 (mean of twenty-four estimations, S.E.M. 0·001). It should be emphasized that this correction should only be applied to a turbidity in fresh blood due to lipaemia, and cannot be used in the presence of methaemoglobin. The waveband designated '607 mμ.', although narrow is not strictly monochromatic. R cannot, therefore, be considered an absolute constant, and should be checked with the filters and instrument actually used.

DISCUSSION

The method described occupies less time than the Haldane or Sahli methods, and its reproducibility is shown to be high. The overall error depends on a number of individual errors, and is calculated as the root of the sum of their squares: (1) The systematic error in the calibration of the absorptiometer. This depends on a neutral filter whose density is given by the National Physical Laboratory to $\pm 0{\cdot}5\,\%$. (2) The random error of reading the author's absorptiometer. For a density of 0·600 this is $\pm 0{\cdot}2\,\%$ (twice coefficient of variation, based on figures of Nicholas, 1951). (3) The error in sampling, pipetting, diluting and reading the H 602 model Spekker has been shown above to be $\pm 0{\cdot}368\,\%$. (4) The error in calibration of glassware may be large; British Standards (1938*a*, *b*) require only $\pm 3\,\%$ accuracy for the 0·2 ml. Ostwald-Folin pipette or $\pm 1\,\%$ for the same size capillary pipette. Pipettes should therefore be calibrated for content by weighing water, when accuracy to $\pm 0{\cdot}1\,\%$ is attainable. Absorptiometer cells usually used are not guaranteed by the makers to better than $\pm 1\,\%$, but special cells can be obtained from them accurate to $\pm 0{\cdot}1\,\%$ or better. (5) The error in the comparison with the British Standard scale by the National Physical Laboratory is estimated by them to be $\pm 0{\cdot}5\,\%$. Triplication is here considered to have reduced this to $\pm 0{\cdot}29\,\%$. (6) The error in the determination of the extinction coefficient of oxyhaemoglobin corresponding to the British Standard depends on (1), (2), (4) and (5) above, but (2) becomes negligible because of replication, and only the cell need be considered under (4). The error therefore becomes $\pm 0{\cdot}59\,\%$.

In comparing an unknown sample with the British Standard on an unspecified instrument (1), (3), (4) and (6) above are operative, and the error is $\pm 0{\cdot}87\,\%$. In comparing two samples, both by this method, on unspecified instruments (6) need not be considered, and the error becomes $\pm 0{\cdot}64\,\%$. When maximum accuracy is desired in a comparison, and the same instrument and the same glassware can be used, an error of $\pm 0{\cdot}37\,\%$ can be achieved, and can be reduced further by replication.

The possibility of interference from turbidity, carboxyhaemoglobin and methaemoglobin should be considered. It has been shown above that a turbidity due to lipaemia can be approximately corrected for. Carboxyhaemoglobin does not interfere appreciably in physiological quantities, being read as oxyhaemoglobin. The ratio $E_{546\,m\mu.}$ oxyhaemoglobin/$E_{546\,m\mu.}$ carboxyhaemoglobin is 1·041 (mean of sixteen determinations, S.E.M. 0·0014), so at 5 % carbon monoxide saturation the error from this cause will be only 0·2 % of the reading. Methaemoglobin has an appreciably lower extinction coefficient than oxyhaemoglobin, so bloods containing more than minimal quantities of this pigment should not be examined by the method described.

It is suggested that the method here described provides immediately a means whereby any worker may relate his results to the British Standard with an accuracy sufficient for all clinical purposes, and one with another with an accuracy sufficient for most research purposes. It is further suggested that ultimately the British Standard should be redefined in terms of the extinction of oxyhaemoglobin at 546 mμ., with the tristimulus specification of the Haldane colour tube becoming a substandard until such time as the use of the Haldane method could be abandoned altogether.

SUMMARY

1. A precise photoelectric method is described for the determination of haemoglobin in blood, suitable for routine, research or standardization purposes, and depending on the measurement of its extinction as oxyhaemoglobin at the 546 mμ. line of the mercury arc. The latter can be isolated by a special filter combination.

2. On the British Standard haemoglobin scale 100 % corresponds to an extinction in a 1 in 100 dilution of 1·193. By calculation from this value the specific extinction $E^{1\,g./ml.}_{546\,m\mu.}$ is 806, and the molecular extinction $\epsilon_{546\,m\mu.}$ is 53,910.

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The Activation of a Proteolytic Enzyme in Blood by Animal Tissue

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This paper presents an investigation of the activation of the proteolytic enzyme in blood (plasmin, fibrinolysin) by an interaction between its proenzyme (plasminogen, profibrinolysin) and the insoluble tissue activator (fibrinokinase) recently discovered (Astrup & Permin, 1947, 1948; Permin, 1947, 1949, 1950*b*). This reaction appears to be of a stoicheiometrical and not of a catalytic nature (Astrup, 1949).

METHODS AND MATERIALS

The fibrin plate method (Permin, 1947, 1949, 1950*a*; Astrup, 1948), as modified by Astrup, Crookston & MacIntyre (1950) was used for measuring the activity of the enzyme. Activities were recorded as the product of two perpendicular diameters of the lysed zones, usually the mean value of three single determinations being taken. Although the proportionality between enzyme concentration and this product, as recorded by Permin (1950*a*), could not be confirmed (Astrup *et al.* 1950), it has been found convenient to use it as an estimate of the enzymic activity, when carrying out comparative investigations. The recorded results, therefore, do not represent a precise quantitative estimation of the enzyme concentration, more especially at higher activities. The applicability of the fibrin plate method, as a general method of estimating fibrinolytic enzymes, is treated in detail in papers now under preparation.

Ox fibrinogen, bovine plasminogen and fibrinokinase from pig heart were prepared as previously described (Permin, 1950*a*, *b*; Astrup *et al.* 1950). Ox thrombin was obtained from Løvens kemiske Fabrik, Copenhagen.

RESULTS

The activation time. The time needed for activation was determined in the following way

Tubes containing 10 ml. each of bovine plasminogen and a few drops of toluene were slowly rotated at room temperature for different lengths of time. One series (*A*) contained 50 mg. of fibrinokinase, the second (*B*) 250 mg. in each tube. The insoluble fibrinokinase was removed by centrifugation and the samples stored in the ice box until all samples could be tested. Drops were then placed on the fibrin plates and the results recorded after 20 hr. at 32° (Table 1). A sample of plasminogen and samples containing fibrinokinase plus saline or buffer were rotated for 24 hr. at room temperature as controls. They did not show any activity.

The experiment shows that maximal activation is obtained after about 4 hr. The activity obtained at the lower concentration of fibrinokinase is only a fraction of that obtained with the higher concentration, and is not increased significantly by increasing the reaction time. Moreover, the large amount (*B*) yields a much higher activity after only 30 min. than the maximal activity obtained with the small amount (*A*). It may be concluded that a small amount of fibrinokinase is unable to produce as much activation in a plasminogen sample as a larger amount, even if the reaction time is increased. This means that the reaction is not of a catalytic nature, but is one in which a component is used up during the process.

Table 1. *Activation of bovine plasminogen by means of fibrinokinase*

Time of rotation	Diameter products (mm.²)	
	A, 50 mg.	*B*, 250 mg.
Zero	0	0
15 min.	(0)	36, 49, 64
30 min.	(0)	90, 100
60 min.	25, 20, 25	100, 121, 144
2 hr.	49, 42, 42	169, 156, 169
4 hr.	56, 42, 36	196, 182, 182
24 hr.	56, 72, 64	169, 169, 225

It was found in subsequent experiments that the activity gained by increasing the activation time

from 4 to 24 hr. was insignificant for all concentrations of fibrinokinase. In most experiments performed during this investigation the reaction was therefore interrupted after 4 hr.

The results obtained in these preliminary experiments did not conform with our expectations, as the activation of human plasminogen by means of streptokinase is said to be a catalytic reaction (Christensen & MacLeod, 1945). This prompted us, therefore, to undertake a more detailed investigation of the activation process.

Varying concentrations of fibrinokinase. The influence of varying the amount of fibrinokinase on the activity obtained was first studied. Fig. 1 shows

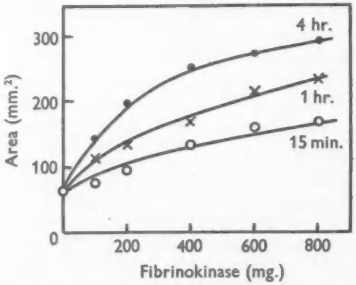

Fig. 1. Activation of plasminogen (10 ml.) by different amounts of fibrinokinase. The plasminogen sample contains a small amount of plasmin formed spontaneously.

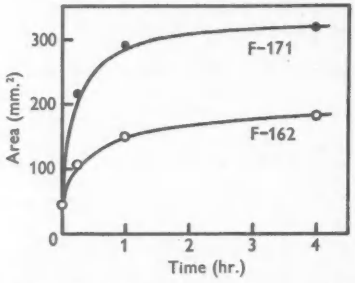

Fig. 2. Activation of plasminogen by two different fibrinokinase samples (400 mg. fibrinokinase/10 ml. plasminogen).

such an experiment, in which 10 ml. samples of plasminogen solution were shaken with fibrinokinase. The activity increases with increasing amounts of fibrinokinase and different preparations of fibrinokinase may produce different activities as shown in Fig. 2. This again is evidence against a catalytic action.

The activation proceeds very rapidly during the first few minutes, and the values obtained after 15 min. of activation are often nearly proportional to the concentration of fibrinokinase (Fig. 1). This is better illustrated by Fig. 3 which compares the activating effect of various quantities of two different samples of fibrinokinase when suspended in 10 ml. plasminogen solution for 15 min. Such curves may be used for comparing the potencies of different samples of fibrinokinase, and Fig. 3 shows that one of the samples (F-2001, 1) is 1·6 times as potent as

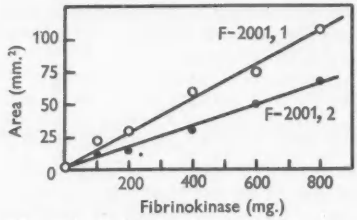

Fig. 3. Comparison of the potencies of two samples of fibrinokinase by measuring the activation of plasminogen after a 15 min. incubation period.

the other (F-2001, 2). The two samples of fibrinokinase behave as if they contained different concentrations of the activating substance. The main requirement of this method of determination is that the solution should contain plasminogen in excess.

Varying concentrations of plasminogen. The preceding experiments suggest that a stoicheiometrical reaction, involving some component of the fibrinokinase preparation, takes place during the activation. The next step was to see if the same were true for the plasminogen and experiments were therefore performed in which the concentration of plasminogen was varied.

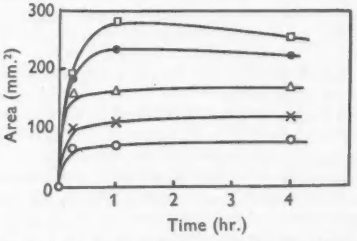

Fig. 4. The time required for complete activation of different concentrations of plasminogen (10 ml.) by a constant amount of fibrinokinase (800 mg.). ○—○, 10%; ×—×, 20%; △—△, 40%; ●—●, 70%; □—□, 100%.

In Fig. 4 is shown the result of an experiment in which 10 ml. of different dilutions of plasminogen were treated for varying periods with a large amount (800 mg.) of fibrinokinase. It is seen that the activity increases during the first hour and then remains almost constant for the next few hours for all concentrations of plasminogen used.

When the amount of fibrinokinase as well as the concentration of plasminogen is varied curves as in Fig. 5 are obtained. It is evident from these experiments that the activity obtained by the interaction between fibrinokinase and plasminogen depends on the concentrations of both the interacting components. The curves have the appearance of a stoicheiometrical reaction moving to an equilibrium.

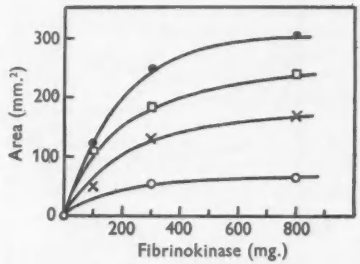

Fig. 5. Activity of varying concentrations of plasminogen (10 ml.) after 4 hr. incubation with different amounts of fibrinokinase. ○—○, 10%; ×—×, 30%; □—□, 60%; ●—●, 100%.

Repeated treatment of plasminogen with fibrinokinase. The next experiment was to treat a plasminogen solution repeatedly with fresh fibrinokinase. If the activation involves a reversible reaction this procedure would force the reaction in the direction of complete activation.

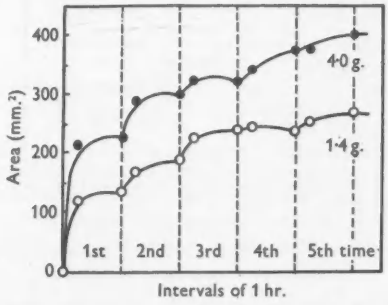

Fig. 6. Repeated treatment of a sample of plasminogen with fresh fibrinokinase at two different concentrations (1·4 and 4·0 g./100 ml.).

To 50 ml. plasminogen solution was added fibrinokinase, and samples were removed after 15 min. of activation. After 60 min. the fibrinokinase was removed by centrifugation, and a sample of the active solution put aside. The remaining solution was then again treated with the same proportion of fibrinokinase. This procedure was repeated five times with each plasminogen solution, the total time for each treatment being 60 min. The measurements are shown in Fig. 6.

It is evident that the addition of fresh fibrinokinase increases the activity of the partially activated plasminogen solution, but that the initial increment in activity is the largest. It had been hoped that the results obtained with 1·4 g. fibrinokinase/100 ml. plasminogen solution would approach the curve for 4·0 g. fibrinokinase/100 ml., when a number of treatments were carried out. Neither in this nor in other experiments could this be obtained under the present experimental conditions.

Similar experiments were carried out using two different samples of fibrinokinase instead of different amounts of the same fibrinokinase sample. The results corresponded with those presented in Fig. 6, and show that the activity depends on the quality as well as on the amount of the fibrinokinase used. Similarly, the activity obtained by repeated treatment of a plasminogen solution with fresh fibrinokinase depends upon the concentration of plasminogen.

Repeated treatment of fibrinokinase with plasminogen. The experiments just mentioned confirm the previous experiments, in that the activity obtained depends upon the concentration of fibrinokinase as well as on that of plasminogen, thus indicating a possible equilibrium reaction between these substances. If this hypothesis is true the repeated treatment of fibrinokinase with fresh plasminogen should yield eventually a product unable to produce activation of a new plasminogen sample.

Fibrinokinase (250 mg.) was treated with 25 ml. of plasminogen solution. A small sample of the solution was removed after 15 min. After 60 min. the fibrinokinase was removed by centrifugation. The solution was kept for activity determination and the fibrinokinase sediment was treated with a fresh volume of 25 ml. plasminogen solution in the same manner. Five such consecutive treatments were carried out. The results presented in Fig. 7 show two different series with 250 and 500 mg. of fibrinokinase respectively. The plasminogen solution used in this experiment had a small spontaneous activity.

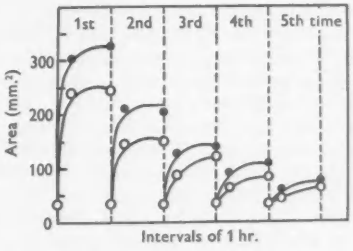

Fig. 7. Repeated treatment of two different amounts of fibrinokinase with fresh plasminogen solution. ○—○, 250 mg. fibrinokinase; ●—●, 500 mg. fibrinokinase.

It is immediately seen that the activating properties of the fibrinokinase sample are completely

lost after repeated treatment with a plasminogen solution. Similar results were obtained when, instead of two different concentrations of the same fibrinokinase sample, two samples of different potencies were used in equal amounts. The simplest conclusion to be drawn from these experiments is that a component of the fibrinokinase reacts with plasminogen during the activation process and is used up in this reaction.

Treatment of plasmin with fibrinokinase. If the conversion of plasminogen to plasmin by fibrinokinase is a reversible reaction, then the treatment of a fully active plasmin solution with a sample of partly inactivated fibrinokinase should produce a decrease in plasmin activity. Therefore the following experiment was performed.

Fibrinokinase (500 mg.) was treated four times for 1 hr. with a plasminogen solution (25 ml.), after which the fibrinokinase had lost most of its activating power. After removal by centrifugation it was kept in saline (+ toluene) at 0° until the next day, when the sample was again centrifuged and washed once with saline. The sediment was then added to 10 ml. of a plasmin solution obtained by four treatments of a plasminogen sample with a large amount of the same fibrinokinase, and then kept overnight.

After treatment for 3 hr. the following activities were found: plasmin after activation, 215 mm.², plasmin treated with inactivated fibrinokinase, 136 mm.².

In a similar experiment the plasmin solution was prepared by repeated treatment with a very potent fibrinokinase. The plasmin sample was then treated: (1) with a less active fibrinokinase, and (2) with a sample of the same fibrinokinase as in (1) which had been treated five times with a plasminogen solution until it was practically inactive. The following activities were found: plasmin alone, 337 mm.²; plasmin treated with a less potent fibrinokinase, 237 mm.²; plasmin treated with an inactivated fibrinokinase, 166 mm.².

The less potent fibrinokinase is able to decrease the activity of a plasmin solution produced by treatment with a more active fibrinokinase, and a fibrinokinase sample, treated previously with plasminogen, is able to decrease the activity of such a plasmin sample.

Technical difficulties have hitherto prevented the preparation of large amounts of fibrinokinase-activated plasmin and of the plasminogen-treated fibrinokinase, which are needed for a satisfactory completion of experiments of this kind. The routine preparation of larger amounts of plasminogen has for some time presented unforeseen difficulties. The activities obtained in plasminogen solutions by spontaneous activation very often exceeded those obtained by repeated treatments with even the most active fibrinokinase. It was also found that the activity of a plasmin solution, maximally active following spontaneous activation, could never be increased further by treatment with a potent fibrinokinase, which suggests that the same enzyme precursor is involved in the two processes. On the contrary, it was observed that the activity could be reduced by treatment with fibrinokinase. The impression was obtained that it was not possible to produce total activation of a plasminogen solution

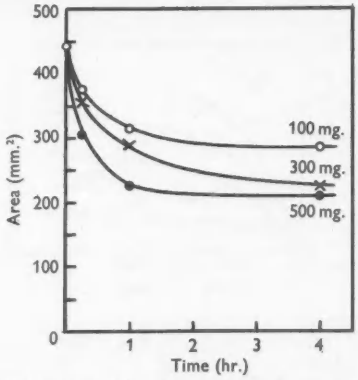

Fig. 8. Treatment of plasmin with fibrinokinase for varying lengths of time.

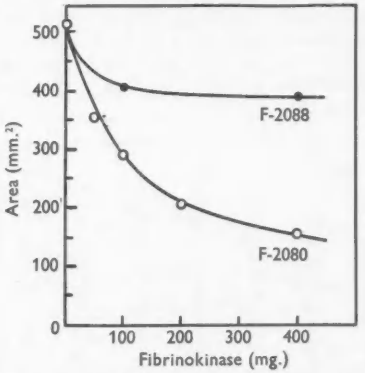

Fig. 9. Treatment of plasmin with two different samples of fibrinokinase in varying concentrations.

with the different samples of fibrinokinase, and that even the best of these might consist partly of already inactivated fibrinokinase. It was therefore decided to investigate more closely the interaction between samples of fibrinokinase and spontaneously activated plasmin.

Such an experiment is presented in Fig. 8. The sample of plasmin used here had a high activity produced by spontaneous activation. Samples of 10 ml. were treated with 100, 300 and 500 mg. of fibrinokinase, and the activities measured after 15 min., 1 and 4 hr. The curves presented in Fig. 8

are almost mirror images of the activation curves obtained by treating plasminogen with fibrinokinase for varying lengths of time (Fig. 2). Complete equilibrium is obtained in 4 hr. Similarly, the curves obtained by treating spontaneously activated plasmin with different amounts of fibrinokinase for a period of 4 hr., as presented in Fig. 9, are mirror images of the corresponding activation curves (Fig. 1). The quality of the fibrinokinase determines the extent of inactivation. Of the samples used in this experiment F-2088 showed high activating power towards plasminogen, while F-2080 was less active (Fig. 11*a*). On the other hand F-2080 has greater inactivating power towards plasmin than F-2088 (Fig. 9).

In another experiment fibrinokinase (F-2080) was treated repeatedly with a spontaneously activated plasmin solution. The results (Fig. 10) show that the

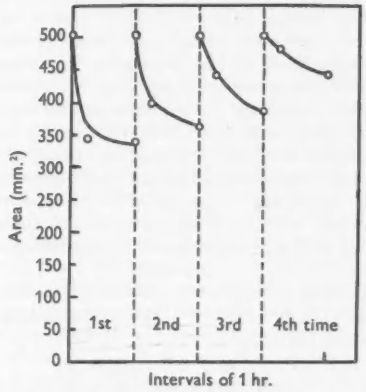

Fig. 10. Repeated treatment of fibrinokinase with plasmin (400 mg. F-2080 to 10 ml. plasmin solution).

ability to inactivate the plasmin solution is slowly decreased after repeated treatment with plasmin. The decrease is perhaps less rapid than would be expected from the corresponding activation curves (Fig. 7).

Behaviour of partially activated plasminogen solutions. When three different samples of fibrinokinase of different potency (Fig. 11*a*) were allowed to act on a plasminogen sample which showed a partial spontaneous activation, the curves presented in Fig. 11*b* were obtained. The most active fibrinokinase (F-2088) still produced a further slight activation. The less active fibrinokinase (F-2020) produced a slight inactivation and the almost inactive fibrinokinase (F-2080) produced a rapid inactivation of the plasmin present.

When a mixture was made of plasminogen and plasmin activated spontaneously or by treatment with a potent fibrinokinase, and then treated with the same three samples of fibrinokinase, the results presented in Fig. 12 were obtained. The potent fibrinokinase (F-2088) is still able to increase the activity of the mixture, while the less potent F-2020 produces a slight activation, and the almost inactive F-2080 a slight inactivation. In further experiments mixtures were prepared in which F-2020 caused no activation or inactivation. This suggests that mixtures of plasmin and plasminogen may be prepared which are in equilibrium with a given sample of fibrinokinase.

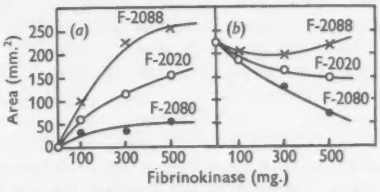

Fig. 11. Action of three different samples of fibrinokinase on plasminogen (*a*) and on a sample of plasminogen partially active as a result of spontaneous activation (*b*). In each experiment 10 ml. solution were treated for 4 hr. with the amount of fibrinokinase recorded.

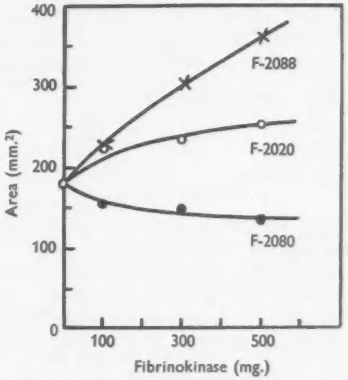

Fig. 12. Action of three different samples of fibrinokinase on a mixture of plasmin and plasminogen.

Spontaneous activation of plasminogen. In several of the experiments mentioned above, samples of spontaneously activated plasmin were used. For the understanding of the activation process the occurrence of a spontaneous activation of plasminogen is of considerable importance.

The samples of plasminogen prepared varied to a considerable extent in stability. Some would keep for long periods of time when stored in the ice box. Some were slowly transformed into a potent plasmin in the course of 1 or 2 months. Others needed only a few weeks or even days for this transformation. In a few cases the plasminogen was active immediately after its preparation from ox serum. The reason for this great variation in the stability has not yet been

found, though a large amount of work has been carried out because the problem is of importance in the routine preparation of plasminogen.

Since a spontaneous transformation of plasminogen into plasmin occurs, the samples must contain all the components needed for the formation of plasmin from plasminogen. The plasmin formed in these samples deteriorates slowly and the solutions finally become inactive. In Table 2 examples are given of the activity variation of samples kept in the ice box. Minor variations in activity are due to variations in the test. It is evident from this table, too, that plasminogen is not activated by the presence of plasmin, thus excluding any simple autocatalytic reaction.

Table 2. *Spontaneous activation of plasminogen*

(Activity recorded as product of two diameters of the lysed zones. Figures in brackets show activity after treatment of 10 ml. solution with 400 mg. fibrinokinase.)

Age of preparation (days)	Activity of preparation ($mm.^2$)			
	F-1030	F-1016	F-1009	F-1034
0	17	23	0 (240)	65 (122)
4	—	114	81 (283)	—
8	4	—	—	—
11	—	169	—	—
13	—	225	64	—
31	—	—	—	390 (156)
36	431	—	—	—
39	—	—	215	—
65	—	—	117	—
68	236	—	—	—

DISCUSSION

In a search for an explanation for the evidence presented here the following points emerge.

Plasminogen and plasmin may be kept in the same solution without any evidence of interaction (Fig. 12 and Table 2). This indicates that the activation of plasminogen to plasmin is not an autocatalytic reaction.

The total amount of active enzyme obtained after bringing the reaction to completion seems to depend to almost the same extent on the concentration of plasminogen as on the amount of fibrinokinase. Extending the reaction time never brought the activity of the samples containing small amounts of fibrinokinase to the same level as that reached in a shorter time with the larger amounts of fibrinokinase. The activation process, therefore, appears to be symmetrical with regard to plasminogen and fibrinokinase, and is most easily described as a stoicheiometrical reaction proceeding to an equilibrium

$$\text{plasminogen} + \text{fibrinokinase} \rightleftharpoons \text{plasmin} + \text{inactive fibrinokinase}. \quad (1)$$

As fibrinokinase is insoluble, and the plasminogen solution may be activated spontaneously or by treatment with chloroform without the addition of other components, it seems unlikely that fibrinokinase releases a soluble component which combines with the plasminogen. The simplest explanation is that fibrinokinase removes reversibly a blocking substance from plasminogen, thus converting it into plasmin. Equation (1) may consequently be divided into the following two equations, where c denotes concentrations:

$$\underset{c_1}{\text{Plasminogen}} \rightleftharpoons \underset{c_2}{\text{plasmin}} + \underset{c_3}{\text{blocking substance}}; \quad (2)$$

$$\underset{c_6}{\text{Blocking substance}} + \underset{c_5}{\text{fibrinokinase}} \rightleftharpoons \underset{c_4}{\text{inactive compound}}. \quad (3)$$

The blocking substance then acts as a specific inhibitor of plasmin, converting it into plasminogen. This inhibitor is not identical with the plasmin inhibitor (antiplasmin) present in serum, as the compound between antiplasmin and plasmin is easily split by a simple ammonium sulphate precipitation, while plasminogen may be purified by repeated treatment with ammonium sulphate without being activated. The spontaneous activation and the activation produced by chloroform may consist of a denaturation of the blocking substance released according to equation (2). If this substance is removed, the reaction proceeds to the right, resulting in the transformation of plasminogen into plasmin.

Equations (2) and (3) describe two dissociation processes for which the following dissociation constants may be derived:

$$k_2 = \frac{c_2 c_3}{c_1}; \quad k_3 = \frac{c_5 c_6}{c_4}. \quad (4)$$

From the experiments it appears possible to prepare mixtures of plasmin and plasminogen, which are in equilibrium with fibrinokinase samples of different potency. This indicates that the samples may contain a mixture of free fibrinokinase and fibrinokinase already saturated with the blocking substance and transformed into the inactive compound. If fibrinokinase and inactivated fibrinokinase are supposed to be present in the solid phase in equilibrium with the saturated solution, then c_4 and c_5 will appear as constants. Consequently, c_6 will be constant, and when equilibrium between reactions (2) and (3) is reached, this will be true for c_3. Therefore

$$c_2 = c_1 . \text{constant}. \quad (5)$$

This equation does not include any relation between the amount of plasmin present and the composition of the fibrinokinase sample in terms of its content of active and inactive fibrinokinase. The experiments give the impression of a competition between fibrinokinase and plasmin for the blocking

substance, which for the insoluble fibrinokinase has the characteristics of an adsorption reaction.

If Langmuir's adsorption isotherm is assumed to be valid for the binding of the blocking substance to the insoluble fibrinokinase, results are obtained which qualitatively are in conformity with the experimental data. The concentration (c_6) of the adsorbed material left in solution is related to the saturated fraction α of the adsorbing material (fibrinokinase) by the following formula, where K is a constant:

$$c_6 = \frac{\alpha K}{1-\alpha}. \qquad (6)$$

When equilibrium is reached for the total reaction $c_6 = c_3$, and $\frac{\alpha}{1-\alpha} = \frac{c_4}{c_5}$. We then get

$$k_2 = \frac{c_2}{c_1}\frac{c_4}{c_5}K \quad \text{or} \quad \frac{c_2}{c_1} = \frac{c_5}{c_4}.\text{constant}. \qquad (7)$$

This means that in such a mixture the ratio between the concentrations of plasmin and plasminogen is proportional to the ratio between the amounts of free fibrinokinase and inactive compound. The equations furnish a qualitative explanation of the interrelationships observed in mixtures of plasmin and plasminogen when samples of fibrinokinase are added, though the nature of the reactions involved is still obscure.

It may be mentioned that the same conditions of equilibrium are obtained if it is postulated that a component splits off from the fibrinokinase and interacts with plasminogen to form plasmin. The present experiments do not allow a distinction to be made between the two possibilities under consideration.

It appears that in the organism a completely controlled equilibrium for a proteolytic system exists. The fibrinokinase in the tissue may be partially saturated with a blocking substance in equilibrium with plasmin. This blocking substance seems to be of a rather specific nature interacting with a specific activator and enzyme. The probable occurrence of a process of this nature in the activation of proteolytic enzymes is of considerable interest, because the activation processes studied previously —with the exception of the activation by means of certain metal ions and the thiol-disulphide activation—all seem to be of a catalytic nature. A similar reaction was proposed 25 years ago by Waldschmidt-Leitz (1924) to explain the activation of trypsinogen by enterokinase. This was shown later by Northrop, Kunitz & Herriott (1948) to be erroneous, when they prepared crystalline proteolytic enzymes, although they were able, in addition to the isolation of crystalline enzymes and enzyme precursors from the pancreas, to isolate crystalline proteolytic inhibitors.

For the plasminogen-plasmin transformation it is too early to draw any definite conclusions, although the experiments presented here are most easily explained on the basis of a reversible reaction. Some crucial experiments, planned to give the final answer to the question, are still to be performed. They have been delayed, however, because they need large amounts of plasminogen and plasmin. The routine preparation of these substances in sufficient quantities and their purification have caused considerable trouble without it being possible to find any definite reasons for this difficulty. Troubles are also caused by the instability of the active enzyme.

The results presented here do not necessarily contradict the suggestion of Christensen & MacLeod (1945) that the action of streptokinase on human plasminogen is of a catalytic nature.

SUMMARY

1. The amount of plasmin formed by the interaction of fibrinokinase with bovine plasminogen depends both on the amount of fibrinokinase and on the amount of plasminogen.

2. The activity of potent plasmin solutions may be reduced by samples of fibrinokinase.

3. Mixtures of plasmin and plasminogen may be prepared which are not influenced by samples of fibrinokinase.

4. An equilibrium reaction between bovine plasminogen and fibrinokinase is suggested for the formation of plasmin. The probable nature of such a process is discussed.

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Electrical Stimulation *in vitro* of the Metabolism of Glucose by Mammalian Cerebral Cortex

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Glucose is the main substrate of the brain *in vivo* and is converted almost entirely to carbon dioxide, water and lactic acid. The rates of both the oxidation and the glycolysis increase on stimulating the brain *in situ* by electrical and also by some other means. Following our development of apparatus for electrical stimulation of portions of separated tissues (McIlwain, 1951), we have now examined the changes brought about by stimulation in the use of glucose by the cerebral cortex, measuring both the loss of this substance and the formation of its products.

EXPERIMENTAL

Media; procedure. Glycylglycine saline was that of McIlwain, Buchel & Cheshire (1951). Details given by McIlwain (1951) were followed, unless otherwise stated, with respect to phosphate and bicarbonate salines, manometric measurements, preparation of tissue, tissue-holding electrodes, and electrical stimulation. The experimental procedure described there was also followed, with the following additions. At the end of a manometric experiment the vessels were promptly removed from the thermostat and a portion (usually 0·5 or 1 ml.) pipetted into $CuSO_4$ for determination of lactic acid according to Barker & Summerson (1941). Another portion (usually 0·2 or 0·5 ml.) was taken for glucose according to Hagedorn & Jensen (see Hawk, Oser & Summerson, 1947).

Vessels and reagents in relation to lactic acid formation. Any failure in aeration increases lactic acid formation by brain tissues. Most experiments were carried out in vessels similar in size and shape to ordinary manometric apparatus, which is known to give adequate aeration. The virtues of the normal design were shown when, to alter electrical conditions, differently shaped vessels were used. In some of these, increased lactic acid was found in the absence of any stimulation. They were not further used and are not described. In all experiments recorded below, the stimulated and control tissues have been in vessels of closely similar shape and size.

In many experiments before and during the present studies, the formation of lactic acid by brain cortex slices has been found to be independent of moderate changes in the weight of tissue/unit volume of fluid. Commonly about 20 mg. fresh wt./ml. has been used (McIlwain & Grinyer, 1950). During some experiments, however, lactic acid formation per unit weight of tissue has been found to increase with increasing fluid/tissue ratio, being increased for example by 50% when the ratio was trebled. This would introduce errors in computing the acid formation from the CO_2 exchange in bicarbonate salines. The reason for this occasional behaviour was not definitely established, but was possibly related to the quality of the distilled water employed. Water redistilled from all-glass apparatus, and whenever possible a uniform tissue/fluid ratio, have therefore been employed in the present experiments.

Tissue-holding electrodes and lactic acid formation. Previous studies showed a variety of electrode types to have little effect on the respiratory rates of slices fixed in them. Change in lactic acid formation has, however, been observed with some electrodes. An instance is given in Exp. 2, Table 1. Other increases, in gauze electrodes, have been 22 and 24% (electrodes *B* in phosphate and bicarbonate salines respectively). On the other hand, some wire electrodes led to lowered lactic acid formation. Thus electrodes *C*, in phosphate-glucose saline with vessels *A*, led to a fall of 30%. The relative instability of aerobic glycolysis is generally realized (Warburg, 1930).

The changes quoted above are opposed to, or are small in comparison with, those reported below to result from applied potentials. Nevertheless, it was necessary to eliminate this factor, and all observations on the effects of stimulation on lactic acid formation have therefore been based on comparisons between slices all of which have been held in the same type of tissue-holding electrodes, but of which some only were stimulated.

Effect of current on medium alone. We have noted (McIlwain, 1951) that under some conditions—avoided in these experiments—the passage of currents suitable for stimulating nervous tissues could result in acid formation from glucose. Although it did not seem likely that lactic acid would be formed, the method for lactic acid determination was not entirely specific, and it was therefore necessary to determine lactic acid in solutions through which currents corresponding to the experimental ones were passed, in the absence of tissue. These never showed any appreciable quantities of apparent lactic acid. Results in two media are quoted in Table 1, Exps. 1 and 7. Other experiments gave: electrodes *A* with glycylglycine glucose saline, $<4\%$ of that by the slices; electrodes *A*, in phosphate-glucose saline, $<6\%$ of that of the slice.

RESULTS

Lactic acid formation

The formation of lactic acid in a variety of experimental media has been determined chemically at the end of different periods of metabolism. In addition, the promptness of glycolytic response to stimulation has been judged in bicarbonate salines, and the course of acid formation computed by applying Warburg's indirect method to obtain the CO_2 and O_2 exchange.

Table 1. *Lactic acid formation in tissue-holding electrodes, and on stimulation*

(All media contained glucose and Ca salts. Experiments were at 37° except no. 9, which was at 39°. With phosphate- and glycylglycine-buffered media, O_2 was used in the gas phase and NaOH paper in a centre well; with bicarbonate-buffered media, O_2–5% CO_2 was used in the gas phase. Electrodes were of silver. Results in Exps. 1*b* and 7*b* were calculated assuming a tissue weight equal to that of Exps. 1*a* and 7*a* respectively. Calculation of the percentage increase in lactic acid formation is described in the text. The increase in respiration, where quoted, was directly observed from 5 min. manometric readings. The voltage quoted in describing condenser pulses is that taken from a battery; the peak voltage when measured from the vessel leads by a cathode-ray tube was about 80% of that applied. The resistance of the vessel was such that the voltage of each pulse fell to one-third its original value in about 1 msec.)

	Metabolic arrangements				Electrical arrangements			Lactic acid produced			
Exp. no.	Vessel type (see Fig. 3, McIlwain, 1951)	Medium*	Slice† fixed unless stated as free	Duration of experiment (min.)	Duration of stimulation (min.)	Stimulation‡ (V.)	Electrode type	Unstimulated (μmol./g./whole exp.)	Stimulated (μmol./g./whole exp.)	Increase during stimulation (%)	Respiration increase during stimulation (%)
1*a*	*A*	P	*o*	155	Three 21 min. periods	1·5, 3, 5 a.c.	*A*	76·5	104	87	Mean, 63
1*b*	*A*	P	None	155	Three 21 min. periods	1·5, 3, 5 a.c.	*A*	<6	<6	—	—
2*a*	*A*	B	*i*	195	40–80; 126–166	2·5 a.c.	*B*	83	165	244	—
2*b*	*A*	B	*i*	195	None	—	None	68	—	—	—
3*a*	A^1	G	*i*	40	5–40	2·9 a.c.	*D*	56	125	141	120
3*b*	A^1	G	*o*	40	5–20	2·9 a.c.	*D*	56	115	210	About 100
4*a*	A^1	P	*i*	221	48–91; 124–191	1·6, 2·5 a.c.	*A*	58	102	151	16, 43
4*b*	A^1	P	*o*	221	48–91; 124–191	1·6, 2·5 a.c.	*E*	43	56	60	9, 24
5*a*	A^1	P	*a*	220	54–94; 126–186	1·7, 2·5 a.c.	*C*	49	95	207	18, 53
5*b*	A^1	P	*o*, free	220	54–94; 126–186	1·7, 2·5 a.c.	*C*	—	46	0	—
6*a*	A^1	B	*o*	180	61–101; 141–180	2·3, 2·5 a.c.	*D*	56	80	80	—
6*b*	A^1	B	*o*, free	180	61–101; 141–180	2·3, 2·5 a.c.	*D*	—	59	5	—
7*a*	*B*	B	*i*	215	Four 30 min. periods	1·2, 1·9, 2·9, 3·9 a.c.	*B*	102	185	151	—
7*b*	*B*	B	None	215	Four 30 min. periods	1·2, 1·9, 2·9, 3·9 a.c.	*B*	<4	<4	—	—
8*a*	A^1	P	*a*	155	33–57; 81–106	6, 24 *C*	*D*	68	100	150	78, 100
8*b*	*B*	P	*i*	155	57–81; 107–131	6, 24 *C*	*B*	92	111	67	—
9	A^1	P	*i*	130	66–98; 102–125	24 *C*	*D*	79	121	127	57

* P, phosphate; B, bicarbonate; G, glycylglycine. † *o*, outer; *i*, inner. ‡ a.c., alternating current; *C*, condenser pulses.

Increase on stimulation. It was easy to show large increases in lactic acid formation associated with electrical stimulation. Results are quoted in Table 1.

In most of these experiments stimulation was not applied for the whole experimental period. The effect of stimulation has therefore been calculated by assuming that in the experimental vessel the additional lactic acid was all formed during the period of stimulation. This gives results implying that lactic acid formation has increased to between 1·5 and 3·5 times its original rate during the various types of stimulation applied.

Kinetic experiments are described below. Here it may be noted that lactic acid formation by brain-tissue slices in general, independently of stimulation, is commonly not uniform, but greater in the first 20 min. or so of an experiment; and also that this calculation assumes a fairly prompt cessation of the increased rate of lactic acid formation. Even apart from the results of such calculations, however, values show a doubling of lactic acid to result from stimulation in some experiments. This occurred with inner and outer cortex slices, and in a variety of media and electrode arrangements which are listed in Table 1. It occurred as a result of the stimuli which had been found effective in causing increase in respiration, with sine-wave a.c. or with condenser pulses. Some forty experiments of this sort have been carried out, and in no case has a result opposed to those of Table 1 been encountered.

Effect of stimulation without tissue being fixed in electrode. It was feasible that the current involved in stimulation might convert a normal metabolic product of the slice into lactic acid. To examine this possibility lactic acid was determined at the end of experiments in which slices had been metabolizing (*a*) free, and (*b*) attached to electrodes; similar potentials were applied to each electrode and similar currents were found to flow in the vessels. Exps. 5 and 6 of Table 1 show that greatly increased lactic acid formation resulted only when the slices were attached to the electrodes. A close relationship to the stimulating electrode is thus necessary for increased glycolysis, as for increased respiration (McIlwain, 1951).

Manometric observations on the course of acid formation in bicarbonate-containing solutions. These observations have been used to show the promptness and course of the metabolic response to stimulation, and also to give information concerning the products. A relatively simple type of experiment suffices to give information on the first points, as is shown in Fig. 1. This gives the gas exchange caused by stimulated and unstimulated cortex slices in a bicarbonate-containing medium in equilibrium with O_2–5 % CO_2. The medium and slices were in vessels with no absorbing agent. Stimulation (vessel *C*, Fig. 1) is seen to have caused a very prompt alteration in the time course of the change in pressure. The alteration did not occur when the tissue was not attached to electrodes at which the potential change was occurring (curve *B*). The imposed alteration ceased promptly when stimulation was stopped. From experiments such as that of Fig. 1 it can be concluded that the metabolic response to stimulation is maximal within 2 min. of applying the potential gradient and ceases within about the same time. The changes may actually be more prompt and the value stated indicates the conclusions which can be drawn from experiments with ordinary manometric techniques.

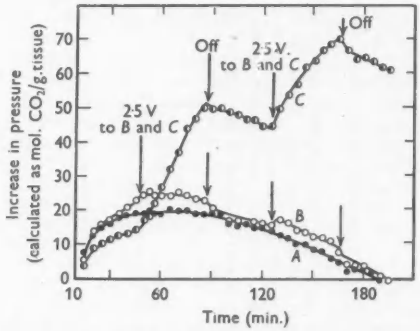

Fig. 1. Change in pressure on stimulating cortex in bicarbonate-glucose saline. Vessels A^1 with electrodes *F*; a.c. at 50 cyc./sec. applied as indicated. Gas phase: 5% CO_2/O_2; 37°; saline about one-tenth of volume of vessel. Line *A*, slice without electrode; *B*, slice floating freely in vessel with electrode to which stimuli were applied; *C*, slice attached to electrode to which stimuli were applied. In order partly to eliminate the effect of different tissue weights and flask volumes, the results have been calculated to apparent μmol. CO_2/g., using the K_{CO_2} of the vessels.

The pressure changes in such an experiment are fairly certainly the net result of O_2 uptake, CO_2 formation, and acid formation from glucose. The O_2 uptake and CO_2 formation, with brain cortex of respiratory quotient about unity (Dickens, 1936*a*), and in vessels and media of the present characteristics, would have as their net effect a gas absorption as the reaction product is the more soluble gas. This explains the fall in pressure in the latter part of the experiment in the absence of stimulation. Increase in pressure we have interpreted as due to acid formation. This has happened at the beginning of the experiment with all slices. Glycolysis in brain cortex slices is known to be active initially and then to fall (for examples and references see McIlwain & Grinyer, 1950). The best explanation of the increase in pressure during stimulation appears to be that it also reflects a higher rate of acid formation. The known

increase in respiration on stimulation would cause greater gas absorption unless the respiratory quotient exceeded unity which is unlikely with glucose as substrate. The increase in gas evolution on stimulation in experiments of this sort has indeed been found to be of the same order of magnitude as the increased lactic acid on stimulation, determined chemically at the end of the experiment. Thus: increased pressure from a slice in a vessel *A*, with electrodes *C*, 63 μmol. CO_2/g.; additional lactic acid, 74 μmol./g. In a vessel *B*, electrodes *B*: additional CO_2, 78 μmol./g. and lactic acid, 97 μmol./g. The discrepancy is of the order to be expected from the fact that changes in O_2 uptake and CO_2 formation on stimulation have been ignored.

A more precise assessment of CO_2 output (displacement and formation) and O_2 uptake has been made by Warburg's indirect method. There are certain difficulties in applying this method to the present experiments. Those relating to the different volumes of solution in the two vessels were obviated, as noted above. More important was the difficulty of ensuring equal stimulation in the two vessels which is necessary for joint calculation of the gas exchange. Potentially, the variables are very large. In practice we have found that most cortex slices cut first, second or third and trimmed so that about two-thirds of their area remains, and fitted as uniformly as possible to electrodes made as similarly as possible, give fairly similar results. In doing this stimuli have been used which approached those giving maximal response. Table 3 gives results from two experiments and the chemically determined lactic acid in the pairs of vessels are seen to be in reasonable agreement.

The course of the gas exchange in one experiment is quoted in Table 2. Stimulation more than doubled the O_2 absorbed and the CO_2 evolved. The stimulated rates of both processes were uniform over the hour of observation. If it is supposed that the respiratory quotient has remained unity, the CO_2 from sources other than respiration can be calculated and compared with the lactic acid determined chemically. This is the most likely source of such extra CO_2. The comparison is made in the last two columns of Table 3. These show approximate agreement, which is taken as indicating that the above assumptions are not greatly in error; the experiments as a whole are limited by the difficulties described in the preceding paragraph.

Table 2. *Increased* CO_2 *and* O_2 *exchange on stimulation*

(Cortex slices of about 60 mg. were used in two pairs of vessels of which one pair was stimulated as described in Table 3, after a period of 16 min. at 37°. An additional vessel was included in the experiment and taken from the bath at this time; it gave the lactic acid formed before the period during which manometric readings were taken. The corrected pressure changes *H* were observed in vessels (μmolar constants K_{O_2}, K_{CO_2}) containing 7 ml. saline, and those *h* in vessels (μmolar constants k_{CO_2}, k_{O_2}) containing 3 ml. The total volume of the vessels was about 12 ml., and it will be seen that these volumes gave adequate differences in the net gas exchange in the vessels. (First pair: k_{CO_2}, 0·0512; k_{O_2}, 0·0441; K_{CO_2}, 0·0409; K_{O_2}, 0·0238. Second pair: 0·0573, 0·0499, 0·0518, 0·0347, respectively.)

Time (min. after putting to thermostat)	*H* (mm.)	*h* (mm.)	O_2 (μmol./g.)	CO_2 (μmol./g.)
	Stimulated vessels			
15	0	0	0	0
30	1	27	− 36	62·5
45	− 6	50	− 76	126
60	−18	68	−114	186
75	−26	83	−145	232
	Unstimulated vessels			
15	0	0	0	0
30	3	4	− 4	12
45	1	8	−23	40
60	2	15	−42	76
75	1	21	−64	113

Table 3. *Stimulation in bicarbonate-glucose solutions*

(Experiments used inner slices of about 60 mg. in vessels *B*, with electrodes *B*. Stimulation was by sine-wave a.c. at the voltage quoted; experimental arrangement and calculation of gaseous exchange was as described in Table 2. The two values for lactic acid refer to vessels containing large and small volumes of saline respectively.)

Exp. no.	Duration (min.) Whole exp.	Duration (min.) Observational period at voltages quoted	Respiration during stimulated period and corresponding times in controls: Rate (μmol./g./hr.)	Respiration during stimulated period and corresponding times in controls: Total uptake (μmol./g.)	CO_2 formation: Rate (μmol./g./hr.)	CO_2 formation: Total evolution (μmol./g.)	Excess of total CO_2 over total O_2 (μmol./g.)	Lactic acid during observational period, determined chemically (μmol./g.)
1*a*	76	—	76	64	126	113	49	47, 68
1*b*	76	16–76, 2·8 V.	150	145	222	232	87	99, 96
2*a*	62	—	68	43	122	66	23	21, 18
2*b*	61	22–62, 3·0 V.	124	75	196	124	49	69, 62

Table 4. *Increased utilization of glucose on stimulation*

(Experiments were with guinea pig-brain cortex at 39° in phosphate-glucose saline with Ca. The initial concentration of glucose was, however, decreased to about M/300. Vessels A^1 and electrodes D were used with 5 or 8 ml. of the saline and 15–20 mg. tissue/ml. Condenser pulses were at 100/sec. and a.c. at 50 cyc./sec. Observation of respiration began at 7–10 min. after placing in a thermostat and no correction has been made for the unmeasured respiration.)

Exp. no.	Treatment	Duration (min.) Metabolism	Duration (min.) Stimulation	Glucose at end of experiment (μmol./vessel)	Glucose used (μmol./g.)	Lactic acid formed (μmol./g.)	Respiration (μmol./g.)	Glucose corresponding to lactic acid and respiration (see text) (μmol./g.)
1*a*	None; no slice	—	—	24·9	—	—	—	—
1*b*	Slice; no stimulation	180	—	17·5	64	—	216	—
1*c*	Slice; 3·2 V.	180	60	17·4	71	—	240	—
1*d*	Slice; 4 μF., 24 V.	180	120	15·1	85·5	—	290	—
2*a*	None; no slice	—	—	26·2	—	—	—	—
2*b*	Slice; no stimulation	160	—	18·7	70	72	167	64
2*c*	Slice; 3·1 V. a.c.	160	150	15·6	97	141	177	100
2*d*	Slice; 4 μF., 24 V.	160	150	13·3	115	131	252	108

Respiration

The increase in respiration previously found (McIlwain, 1951) has been observed during the present experiments. Instances are recorded in Tables 1–4. In several of these the effect of a given stimulus on lactic acid and respiration could be compared, and in almost all such cases the percentage increase in lactic acid formation was greater than that in respiration.

Glucose utilization

The increases in respiration and in lactic acid formation which have been found to follow stimulation, give expectation of finding increased glucose usage. That this occurs is shown in Table 4. It occurred in response to a.c. and to condenser pulses, and the additional usage was greater the longer the duration of stimulation. In these experiments the condenser pulses were the more effective means of stimulation as judged by respiration and glucose utilization.

We have also examined the balance of metabolism with glucose as substrate. *In vivo*, CO_2 and lactic acid account for almost all the glucose consumed (Himwich & Himwich, 1946). *In vitro*, the tissue slices respire in the absence of added substrate; they are not necessarily maintained in a steady state during an experiment, and substrates may be contributed by the slice. Dixon (1936), however, quotes a conclusion of Dr E. G. Holmes that lactic acid and CO_2 mainly account for the glucose disappearing during respiration of brain-cortex slices in its presence. This was the case during our experiments (Table 4). In assessing these experiments we have supposed all the O_2 consumed to be used in oxidizing glucose in the ratio of 6 O_2:1 glucose; and all the lactic acid to be formed from glucose in the ratio 2 lactic:1 glucose. The glucose corresponding to the observed respiration and glycolysis could then be compared with that observed to be lost, as has been done in the last column. Approximate agreement was then found. In order to measure with reasonable accuracy the glucose disappearing, its total quantity was reduced to about $3{\cdot}3 \times 10^{-3}$ M. This might be expected to increase the tendency of the slices to oxidize autogenous substrates, but the results suggested that this did not occur to any large extent. During stimulation also, the increased quantities of glucose metabolized were largely accounted for by respiration and by the lactic acid formed.

DISCUSSION

The effects of fluctuating electrical potential gradients on the metabolism of portions of mammalian brain can now be compared *in vivo* and *in vitro* with respect to glycolysis as well as respiration. The stimuli in tissue sections under the newly devised conditions increased glucose usage and lactic acid formation. The glycolysis rose from initial values of 15–40 μmol./g./hr. and reached about 100 μmol./g./hr. The increase occurred promptly, being complete within 2 min., which represents the limit of measurement of the present methods; response may have been more prompt.

Glycolysis in the brain *in situ* is also increased promptly by electrical stimulation. A quantitative comparison between the two conditions is more difficult to make. Our experiments have been arranged so that a normal steady state of metabolism has been replaced by a markedly higher steady state. In doing this we have caused the stimulating potentials to affect as much of the tissue as possible, and to cause something approaching the largest change possible in steady state of metabolism. Measure-

ments do not appear to have been made on the result of comparable electrical stimulation *in vivo*, probably because stimulation of a large part of the brain *in vivo* is not likely to lead to stable conditions. The stimulation has major effects on the rest of the organism. Thus, convulsions with large circulatory and respiratory changes take place in the whole animal, as well as vasomotor changes in the brain itself. Increased metabolism of the brain can, however, be detected by arterial-venous difference (cf. Schmidt, Kety & Pennes, 1945) followed by lowered rates in the post-convulsive phase (Kety, Woodford, Harmel, Freyhan, Appel & Schmidt, 1948).

A different technique has given some indications of maximal values for metabolic rates during very brief periods. This consists of analysis of the brain itself, fixed by rapid freezing in liquid air, at intervals of a few seconds during the convulsions which follow general stimulation of the central nervous system. Klein & Olsen's (1947) values for the glucose and lactic acid of the brains of cats in their normal condition and after a 10 sec. convulsive period, suggest changes at the rate of -260 μmol. glucose/g./hr. and $+310$ μmol. lactic acid/g./hr. In making this calculation we have supposed the change to be spread over a period of 23 sec., as Dawson & Richter (1950) showed large changes in related metabolites within a second of applying an electric stimulus, and in Klein & Olsen's (1947) experiments convulsions usually commenced 13 sec. after applying the stimulus. The initial rate may be more rapid than this; rates of change of intermediates measured by Dawson & Richter (1950) were much greater during the first 2 sec. than during the first 20 sec., but these concerned substances (inorganic phosphate, phosphocreatine, and hexose and adenosine phosphates) which can be both formed and used up by the brain. Lactic acid and glucose are not in this category, and the changes calculated on the basis of Klein & Olsen's figures are much higher than any found in the intermediates by Dawson & Richter. It is uncertain whether the changes calculated in the above fashion from Klein & Olsen's values represent the actual rates of reaction during the 23 sec. or whether they represent the increase in those rates (which is the authors' view), for entry of substances to the brain may be restricted. Maximal rates might thus be -370 μmol. glucose/g./hr. and $+340$ μmol. lactic acid/g./hr.

These calculations give rates for glycolysis close to those which have been observed in fortified homogenates. These are between 300 and 450 μmol./g./hr. (Ochoa, 1941; Utter, Wood & Reiner, 1945; Elliott & Henry, 1946; Meyerhof & Geliazkowa, 1947). Indeed the situation in Klein & Olsen's (1947) experiments can be regarded as close to the situation in a homogenate, in that the changes in Klein & Olsen's work probably involved only intracellular substrates. Maximal rates obtained by either of these two methods are much above the values, of up to 100 μmol./g./hr., which we have now found to follow electrical stimulation of the slices.

This is, however, understandable for the following reasons: (1) The high rates *in vivo* last for some seconds only, and those in diluted homogenates are dependent on the addition of reserves of intermediary metabolites which are large in proportion to the tissue weight. (2) The slices *in vitro* must first assimilate the glucose they use; some dilution has taken place in them by their imbibition of fluid from the suspending medium, and some loss of reactants has occurred, e.g. of cozymase (Gore, Ibbott & McIlwain, 1950). Phosphocreatine also is only partly resynthesized. (3) The nutrient saline and conditions of electrical stimulation in our experiments may not be optimal.

It is therefore significant that the increased rates in slices are comparable to the increases which can be induced in them by means other than electrical stimulation. They are greater than the effects of glutamate or absence of calcium salts, previously commented on (McIlwain, 1951), and approach those which can be caused by added substances such as 2:4-dinitrophenol, phenosafranine, and ethyl red (cf. Dickens, 1936*b*). A recent comparison from this laboratory showed that rates up to 125 μmol. lactic acid/g./hr. could be reached in this way (McIlwain & Grinyer, 1950). The electrically stimulated tissue thus appears to be respiring and glycolysing at close to its maximal rates. These are data to be taken into account in any judgement of what may be the counterpart in the brain *in situ* of such a stimulated condition. Judgement cannot, however, be made on the basis of biochemical data alone, and further investigations are in progress.

SUMMARY

1. The changing potential gradients which increase the respiration of slices of cerebral cortex, increase also its rate of utilization of added glucose and its rate of formation of lactic acid.

2. Respiration (the calculation assuming 6 mol. oxygen/mol. glucose) and lactic acid formation account to within 6% for the glucose lost, during stimulation as well as in its absence.

3. The formation of lactic acid, aerobically, can rise from 15 or 40 μmol./g./hr. to 100 μmol./g./hr. The change takes place as promptly as can be detected by the methods employed, i.e. within 2 min. It can be maintained for an hour or more.

4. Response to stimulation *in vitro* is thus in the same direction as in the brain *in situ*; it can be maintained much longer, but does not reach rates equal to those which appear to occur during the first few seconds after stimuli are applied *in vivo*.

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Adenosinetriphosphatase Activity of Brain

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Phosphatases which act on adenosinetriphosphate (ATP) have a wide distribution in nature and are present in many mammalian tissues. DuBois & Potter (1943) described a method of assay of such an enzyme in various tissues including brain. The adenosinetriphosphatase (ATPase) of brain has been relatively little investigated, however, compared with that of muscle.

The important discovery that the contractile protein, myosin, is a highly active enzyme which splits inorganic phosphate from ATP, was made by Engelhardt & Ljubimowa (1939). The relationships between myosin, ATP and various ion concentrations in the contraction of the muscle fibre have been studied in detail mainly by Szent-Györgyi and his colleagues (Szent-Györgyi, 1947). The problem of how far the relationships existing in muscle represent a general principle which is used by other specialized tissues as a source of energy for activity is an interesting one. There is evidence that changes in functional activity are associated with changes in ATP, phosphocreatine and inorganic phosphate in brain (Stone, Webster & Gurdjian, 1945; Klein & Olsen, 1947). The enzymic hydrolysis of ATP has not, however, been shown to be a reaction essential in providing energy for the functional activity of brain.

The presence of an ATP-splitting enzyme in brain and the controlling effect which the concentration of this enzyme has on the rate on anaerobic glycolysis of glucose and fructose by brain homogenates, slices and extracts, has been described in a series of papers by Meyerhof and his colleagues (Meyerhof, 1947; Meyerhof & Geliazkowa, 1947; Meyerhof & Wilson, 1947, 1948, 1949). The importance of an adequate supply of ATP in the maintenance of glycolysis and the effect of the ATPase in brain homogenates and extracts in controlling this process has also been shown by Utter, Reiner & Wood (1945) and Utter (1950).

The present study was made to examine the properties of the ATP-splitting enzyme in brain tissue and to test the effect on it of compounds structurally related to ATP and various drugs which act on the central nervous system. The activity of brain tissue in splitting ATP has been compared with its inorganic pyrophosphatase (PPase) activity (Gordon, 1950).

EXPERIMENTAL

Enzyme preparations. A guinea pig was killed by a blow on the neck. The whole brain was excised rapidly, weighed and homogenized in 10 vol. of ice-cold distilled water, 0·154 M-NaCl solution, 0·5 M-KCl containing 0·03 M-$NaHCO_3$ and 0·2 M-Na_2CO_3, or Krebs-Ringer bicarbonate (Umbreit, Burris & Stauffer, 1949) from which $CaCl_2$ and KH_2PO_4 had been omitted. The homogenate was stored at 0° under toluene at this dilution. For measurement of enzyme activity further dilution of the homogenate to 1 g. tissue in

100 vol. of fluid was made. The tissue extracts obtained as supernatant solution on centrifuging the 1:10 homogenate and the tissue residue resuspended in an equal volume of homogenizing fluid have also been tested for ATPase and PPase activity.

For the experiments in which the effect of various compounds on brain ATPase was being measured, a preparation which contained ATPase relatively free from PPase was desired. This was prepared by homogenizing whole brain in 10 vol. of Krebs-Ringer solution. The tissue was extracted by stirring the homogenate mechanically for 30 min. at 0°. The mixture was then centrifuged, the supernatant removed and replaced with an equal volume of distilled water. After 10 min. stirring, the mixture was again centrifuged and the supernatant replaced with distilled water. Extraction was made for 30 min. This extract contained approximately 50% of the ATPase and 10% of the PPase activity of the initial homogenate.

Measurement of enzyme activity

Adenosinetriphosphatase. BaATP supplied by Boots Pure Drug Co. Ltd. was used to prepare the substrate for the test of ATPase activity. An analysis of the phosphorus content of the BaATP specimen used gave the figures: total P, 11·1%; labile P (10 min. hydrolysis in 1 N-HCl), 6·7%; inorganic P, 1·3%. A weighed sample of the commercial preparation was washed twice with small volumes of ice-cold 0·1 M-acetate buffer (pH 4·0) as suggested by Kornberg (1950) to remove inorganic phosphate present and then dissolved in the minimum amount of 0·1 N-HCl. The washing treatment reduced the percentage of phosphorus present as inorganic phosphate to 0·3% by weight. The Na salt was obtained by passing the acid solution through a column of Na-charged Zeo-Karb 216. The pH of the solution was brought to 7·4 with 0·1 N-NaOH and the molar concentration adjusted to 0·02. The ATP solution (0·5 ml.) was incubated in a stoppered tube with 1 ml. 0·1 M-glycylglycine buffer (pH 7·4) in the presence of $MgSO_4$ and KCl, each at a final concentration of $7{\cdot}7 \times 10^{-3}$ M. The enzyme preparation (0·5 ml.) containing 0·5–2 mg. tissue, dry wt., was then added, the mixture well shaken, a 0·5 ml. sample removed quickly for estimation of phosphate present and the tube returned to the water bath at 37°. The initial and all subsequent samples were added to ice-cold trichloroacetic acid (final concn. 5%). Inorganic phosphate concentration was determined on the trichloroacetic acid filtrate by the method of Lowry & Lopez (1946). Results were calculated as mol. P liberated/hr./mg. tissue, dry wt.

Inorganic pyrophosphatase. The inorganic pyrophosphatase activity of brain preparations (Gordon, 1950) was estimated under the same conditions of pH, buffer and ion activation as the ATPase. $Na_4P_2O_7.10H_2O$ (A.R. quality), free from orthophosphate, was used as substrate at a final concentration of $2{\cdot}5 \times 10^{-3}$ M.

RESULTS

Factors influencing activity of the ATPase

The effects of time of incubation, substrate concentration, enzyme concentration, pH, cation concentration and various buffers, have been measured and the results are shown in Figs. 1–4. The following results were obtained.

Time of incubation. The rate of decomposition of ATP by guinea pig-brain homogenate at pH 7·4 in the presence of $7{\cdot}7 \times 10^{-3}$ M Mg^{++} and K^{++} was linear over a period of 20 min. (Fig. 1*a*). After 20 min. the activity slowly fell, possibly due to inactivation of the enzyme. Preheating the enzyme for 30 min. at 37° reduced its activity by 12%. On the basis of these findings samples taken after 15 min. incubation were used in measurement of enzyme activity.

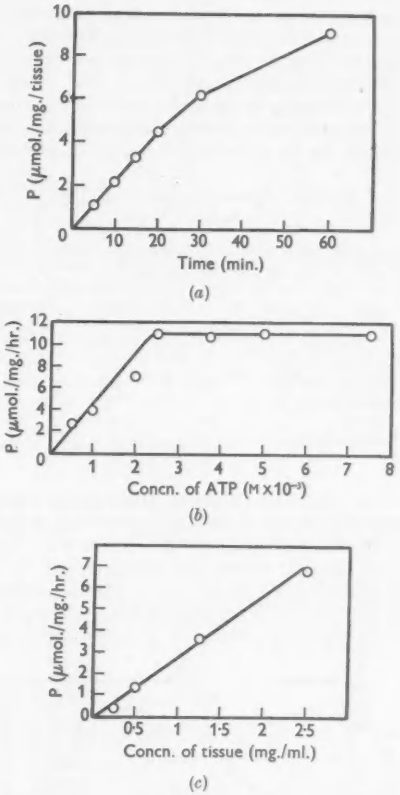

Fig. 1. Effect of (*a*) time of incubation, (*b*) substrate concentration, and (*c*) enzyme concentration on liberation of inorganic phosphate from ATP at 37°. Guinea pig-brain homogenate in 0·1 M-glycylglycine (pH 7·4) containing Mg^{++}, $7{\cdot}7 \times 10^{-3}$ M, and K^{++}, $7{\cdot}7 \times 10^{-3}$ M. In (*a*) and (*c*) ATP concentration is 5×10^{-3} M.

Substrate concentration. The activity of the enzyme was maximal with a substrate concentration of $2{\cdot}5 \times 10^{-3}$ M (Fig. 1*b*). Increase in concentration of ATP to $7{\cdot}5 \times 10^{-3}$ M produced no increase in the rate of liberation of inorganic phosphate. The

concentration of ATP used in the experiments in this study was 5×10^{-3}M.

Enzyme concentration. The amount of phosphate liberated from ATP was directly proportional to the weight of tissue present over the range 0·25–2·5 mg. (dry wt.)/ml. (Fig. 1*c*). Brain homogenates and extracts were diluted so that the concentration of tissue in the incubation mixture was in this range.

Buffers. The effect of several buffers on the rate of enzyme action was studied and the results are shown in Table 1. The rate of liberation of inorganic phosphate was greatest in 0·1M-glycylglycine buffer and this has been used in all experiments. The relatively high rate of activity in glycylglycine buffer may be due to some effect on inhibiting ions or on ion balance exerted by the glycylglycine. In their assay of ATPase activity in various tissues including brain DuBois & Potter (1943) used a diethylbarbiturate

Table 1. *Effect of buffer on ATPase of guinea pig brain*

(Guinea pig-brain homogenate incubated with 5×10^{-3}M-ATP and $7{\cdot}7 \times 10^{-3}$M Mg^{++} and K^+ at 37°.)

Buffer	pH	Rate (μmol. P/mg./hr.)	Percentage of highest rate
Glycylglycine, 0·1M	8·0	7·9	100
Bicarbonate, 0·072M (gassed with 5% CO_2)	8·0	6·0	76
Glycine, 0·1M	8·2	4·7	60
Diethylbarbiturate, 0·1M	8·0	2·5	32

buffer in which, according to Table 1, the enzyme reaction takes place at one-third of the rate of that in glycylglycine buffer.

Optimum pH. When the effect of pH on the enzyme activity was measured there appeared to be two maxima, one at pH 7·4 and the other at pH 8·2 (Fig. 2). This might be due to the presence of more

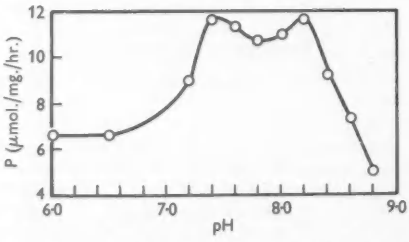

Fig. 2. Effect of pH of medium on ATPase activity of brain at 37°. Medium and ATP concentrations as Fig. 1.

than one enzyme in the brain homogenate acting on ATP or to the existence of a different pH optimum for the splitting of the first and second labile phosphate of ATP.

Number of inorganic phosphate groups liberated and specificity. The number of inorganic phosphate groups split from ATP by brain ATPase was measured at pH 7·4 and 8·2. The result is shown in Fig. 3. There was very little difference in the rate of

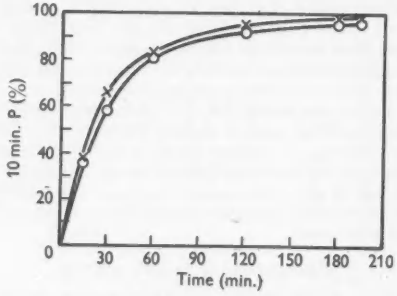

Fig. 3. Number of phosphate groups split from ATP by brain. Medium and ATP concentration as in Fig. 1. Labile phosphate was measured after 10 min. hydrolysis at 100° in 1N-HCl. ×—×, pH 8·2; ○—○, pH 7·4.

hydrolysis of ATP at different pH, and in both conditions two phosphate groups were split from the ATP molecule. One phosphate group was split from ADP when this was used as substrate at pH 7·4. The enzyme did not act on hexosediphosphate and β-glycerophosphate.

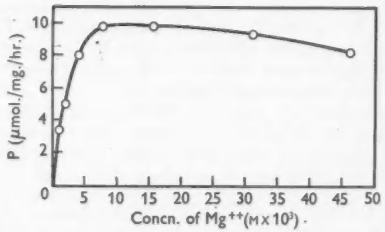

Fig. 4. Effect of Mg^{++} concentration on ATPase activity of brain at 37°. Medium and ATP concentration as in Fig. 1.

Effect of Mg^{++}, Ca^{++} *and* K^+. The activity of the enzyme was slight in the absence of Mg^{++} ions. Optimum activation was given by a final concentration of $7{\cdot}7 \times 10^{-3}$M-Mg^{++} in the incubation mixture (Fig. 4). Both in the absence of Mg^{++} ions and in the presence of the optimum Mg^{++} ion concentration, the addition of Ca^{++} to the medium in concentrations as low as 6×10^{-4}M produced inhibition of the enzyme (Table 2). In the presence of $7{\cdot}7 \times 10^{-3}$M-Mg^{++}, K^+ increased the rate of liberation of inorganic phosphate. The medium used for measuring enzyme activity contained Mg^{++} and K^+ in amounts which gave a final concentration of each of $7{\cdot}7 \times 10^{-3}$M.

These results differ from those of DuBois & Potter (1943), who found that the ATPase of ten different

Table 2. *Effect of* Mg^{++}, K^{+} *and* Ca^{++}, *on ATPase of guinea pig brain*

(Guinea pig-brain homogenate incubated in 0·1 M-glycylglycine with 5×10^{-3} M-ATP at 37°.)

Medium containing final concentration ($M \times 10^{-3}$)			Rate (μmol. P/mg./hr.)
Mg	K	Ca	
0	0	0	1·2
7·7	0	0	5·9
7·7	19	0	9·9
7·7	7·7	0	10·2
7·7	3	0	8·9
7·7	7·7	1·3	4·3
7·7	7·7	0	13·6
7·7	7·7	55	4·3
7·7	7·7	1·3	5·05
7·7	7·7	0·6	7·35
0	7·7	0	2·6
0	7·7	11	1·4
0	7·7	5·5	1·4
0	7·7	2·8	1·3

tissues, including brain, was activated by Ca^{++}. The ATPase of brain they report to be three times more active in the presence of 3×10^{-3} M-Ca^{++} as in its absence. They also found Mg^{++} in concentration above $2{\cdot}4 \times 10^{-3}$ M to inhibit liver ATPase. Other workers, however, support the findings of this study by reporting either Mg^{++} activation or Ca^{++} inhibition or both of ATPase in nervous tissue. (Greville & Lehmann, 1943; Feldberg & Mann, 1945; Epelbaum, Sheves & Kobylin, 1949; Binkley & Olson, 1950).

Effect of various substances on ATPase

Sodium fluoride is known to be a phosphatase inhibitor and has been found to inhibit brain ATPase. The effect of some other common enzyme inhibitors on ATPase has been examined. An attempt to find an inhibitor specific for ATPase has been made by examining the effect of substances structurally related to ATP on the enzyme. Torda & Wolff (1948) studied the effect of some centrally active substances and alkaloids on muscle ATPase. A similar examination on brain ATPase was clearly desirable and this has been made.

Enzyme inhibitors. The effect of various enzyme inhibitors on guinea pig-brain ATPase is given in Table 3. The table also shows the effect of the same concentration of inhibitor on PPase and this will be discussed below. The ATPase appears to be sensitive to low concentrations of substances known to react with thiol groups: copper sulphate, sodium iodoacetate and alloxan. Sodium fluoride is also an active inhibitor. Relatively high concentrations of sodium cyanide are necessary to produce inhibition and the enzyme was almost unaffected by the concentration of 2:4-dinitrophenol used.

Table 3. *Effect of enzyme inhibitors on ATPase and PPase of guinea pig brain*

(Second water extract of homogenized brain tissue in 0·1 M-glycylglycine, pH 7·4, with 5×10^{-3} M-ATP or $2{\cdot}5 \times 10^{-3}$ M-sodium pyrophosphate (PP) in presence of $7{\cdot}7 \times 10^{-3}$ M-Mg^{++} and K^{++} and test substance.)

Substance	Concentration ($M \times 10^{-3}$)	Inhibition (%)	
		ATPase	PPase
NaF	2·5	61	100
NaF	0·5	31	93
NaF	0·25	23	66
$CuSO_4$	0·1	32	87
$CuSO_4$	0·125	33	100
$CuSO_4$	0·01	− 8	4
NaCN	15	19	12
Sodium iodoacetate	2	29	50
Alloxan	1	17	23
Alloxan	10	50	82
2:4-Dinitrophenol	1·6	4	—
2:4-Dinitrophenol	0·3	7	—
2:4-Dinitrophenol	0·06	0	—

Substances structurally related to ATP and substances with action on central nervous system. The action of purine derivatives, barbiturate drugs and several alkaloids in the ATPase activity of brain has been assessed quantitatively. The results obtained with some of the substances tested are given in Table 4.

Table 4. *Effect of various substances on ATPase activity of guinea pig brain*

(5×10^{-3} M-ATP, $7{\cdot}7 \times 10^{-3}$ M-Mg^{++} and K^{+} and test substance in 1·5 ml. 0·1 M-glycylglycine, pH 7·4, at 37°. Second water extract of homogenized brain added, 0·5 ml.)

Substance	Concentration ($M \times 10^{-3}$)	Rate of reaction (as percentage of rate without added substance)
Adenine	5	77
Adenosine	20	84
Guanine	5	98
Xanthine	5	114
Caffeine	6·6	100
Caffeine	1·3	110
Glucose	20	88
Glucose	4	78
Glucose	0·8	89
Glutamate	20	90
Glutamate	4	93
Glutamate	0·8	96
Creatine	20	94
Aneurin	2	91
Aneurin	2	83
Adrenaline	2	83

Of the purine substances tested, adenine produced a slight inhibition of the enzyme at a relatively high concentration. The other purines appeared to have little effect. Meyerhof & Wilson (1949) report a small inhibition of the ATPase of brain homogenate by

octyl and decyl alcohols, which also have narcotic properties. None of the barbiturate drugs, phenobarbitone, soneryl (5-*n*-butyl-5-ethylbarbituric acid), dial (diallylbarbituric acid) or evipan (5-*cyclo*hexyl-1:5-dimethylbarbituric acid) produced any significant inhibition of ATPase in the concentrations 2×10^{-2}M, 4×10^{-3}M, $0{\cdot}8 \times 10^{-3}$M in the present investigations. Diethylbarbituric acid, used in 0·1M concentration as a buffer, did have considerable inhibitory effect (Table 1).

The alkaloids papaverine and narcotine in saturated solution, brucine at 2×10^{-3}M and colchicine at 10^{-3}M and $0{\cdot}4 \times 10^{-4}$M had very little inhibitory action on ATPase. Atropine at $0{\cdot}4 \times 10^{-3}$M produced an inhibition of 17%.

Glucose and sodium glutamate and creatine were added to the reaction medium and produced only a slight inhibition of the enzyme. Meyerhof & Wilson (1949) state that the presence of glucose has an inhibitory effect on ATPase of complete brain homogenate. In the present experiments the rate of glycolysis was not measured, but was probably low due to removal of glycolytic enzymes in the Krebs-Ringer and first water extracts of the brain tissue.

The slight inhibition produced by aneurin and adrenaline are shown in Table 4.

Comparison of ATPase and PPase activity

Early in this investigation the similarity of the conditions of optimum pH, ion activation and effective inhibitors of guinea pig-brain ATPase to the properties of brain inorganic pyrophosphatase described by Gordon (1950) was realized. When the PPase activity of a fresh guinea pig brain homogenized in water, sodium chloride or Krebs-Ringer was measured using the same medium as for ATPase measurement, but sodium pyrophosphate at a final concentration of $2{\cdot}5 \times 10^{-3}$M as substrate, it was found that ATPase and PPase activity of the same preparation were approximately equal. Three possible interpretations of this result were considered: (1) that the enzyme described by Gordon was also capable of acting on organic pyrophosphates; (2) that the ATP being used contained inorganic pyrophosphate as impurity (Bailey, 1949) in sufficient quantity to act as substrate for brain PPase; and (3) that there are two separate phosphatases present in brain, one of which reacts with ATP and the other with inorganic pyrophosphate. Several pieces of evidence indicate that brain contains both ATPase and PPase.

An inorganic pyrophosphate-free sample of ATP was prepared by the method described by Baddiley, Michelson & Todd (1949). Brain homogenates gave the same rate of liberation of phosphate with this preparation as with the non-purified sample.

An acetone-dried preparation of brain was made from guinea pig brain according to the instructions given by Gordon (1950). When the saline suspension of this material was tested for ATPase as well as PPase activity it was found that the ATPase activity was only a fraction (10–17%) of the PPase activity. The fact that acetone drying destroys ATPase activity in brain tissue had previously been noted by Feldberg & Mann (1945) and Meyerhof & Wilson (1948).

Another difference between the enzymic breakdown of the two substrates was the degree of inhibition produced by certain concentrations of inhibitors, notably sodium fluoride and copper sulphate. This is shown in Table 3. It can be seen that concentrations of sodium fluoride, which produced 100 and 93% inhibition of breakdown of inorganic pyrophosphate, affected ATPase by 61 and 31%. Similar distinctions were seen at different concentrations of copper sulphate.

A further way in which ATPase and PPase activity was differentiated was in the distribution of the enzyme activity on these two substrates in the extracts and tissue residue of brain homogenates.

Table 5 shows the ATPase and PPase activity of guinea pig-brain homogenates prepared in water and different salt solutions and of the extracts and tissue residues obtained by centrifuging these homogenates. Guinea pig brain was homogenized in 10 vol. of ice-cold water, 0·154M-sodium chloride, 0·5M-potassium chloride containing 0·03M-sodium bicarbonate and 0·02M-sodium carbonate and Krebs-Ringer bi-

Table 5. *Comparison of ATPase and PPase activity of various homogenate fractions*

(Enzyme incubated in 0·1M-glycylglycine, pH 7·4, with 5×10^{-3}M-ATP or $2{\cdot}5 \times 10^{-3}$M-sodium pyrophosphate (PP) in presence of $7{\cdot}7 \times 10^{-3}$M-Mg^{++} and K^{++}.)

Guinea pig brain homogenized in	Homogenate Rate (μmol. P/mg./hr.) ATP	PP	Ratio ATP/PP	Supernatant Rate (μmol. P/mg./hr.) ATP	PP	Ratio ATP/PP	Residue Rate (μmol. P/mg./hr.) ATP	PP	Ratio ATP/PP
Water	6·5	8·5	0·77	4·85	10·5	0·46	5·85	1·66	3·5
NaCl, 0·154M	7·4	5·3	1·4	3·1	10·5	0·29	8·7	2·06	4·2
Alkaline KCl, 0·5M	4·0	1·5	2·6	2·64	5·8	0·45	3·88	0	—
Krebs-Ringer bicarbonate	5·9	6·1	0·97	3·7	10·7	0·29	6·6	1·5	4·4
	4·0	4·15	0·97	2·4	7·1	0·34	7·0	1·6	4·35

carbonate solution in which there were no calcium salts or phosphate.

The rate at which the homogenates in water, sodium chloride and Krebs-Ringer split ATP and inorganic pyrophosphates was approximately equal. In the sodium chloride and Krebs-Ringer extracts of brain the PPase activity was three times as great as the ATPase activity; in water and potassium chloride the ratio ATPase/PPase was 0·45. In the tissue residues which were resuspended in homogenizing fluid the ATPase activity was approximately four times as great as the PPase activity.

The ATPase/PPase ratio could be further increased if the Krebs-Ringer homogenate was centrifuged and the tissue residue extracted twice with water. In the first water washing more PPase activity and very little ATPase activity was lost. The second water washing contained 30–50 % of the total ATPase activity of the homogenate and only 5–8 % of the total PPase activity. Although the potassium chloride homogenate seemed to show a relatively high ATPase/PPase activity, the ATPase rate was low and the enzyme appeared to be less stable than in the other salt solutions. Potassium chloride was therefore not used in making ATPase preparations.

In the water homogenates, one-fifth of the total ATPase activity of the complete homogenate was found in the extract; with sodium chloride and Krebs-Ringer homogenates only one-tenth of the ATPase activity was in the extract. This fractionation of brain tissue by extraction did not produce any great purification of the ATPase active fraction as can be seen in Table 5. Attempts to purify the enzyme by ammonium sulphate precipitation and acetone precipitation (Binkley & Olson, 1950) did not meet with success as, after dialysis, the activity of the isolated fraction was always extremely low.

DISCUSSION

It would seem from the present study, and from a number of independent reports, that there exists in mammalian brain an enzyme which liberates inorganic phosphate from ATP, and which is activated by Mg^{++} and inhibited by Ca^{++}. What role this enzyme plays in brain tissue is not fully understood. The function which Meyerhof has found it to fulfil, that of controlling anaerobic glycolysis of brain tissue by removing from the medium the energy-rich phosphates of ATP, seems a somewhat negative role for an active enzyme. No direct evidence has been obtained of what use is made of the energy released by the breakdown of ATP with the liberation of inorganic phosphate in brain.

The importance of the calcium-activated and magnesium-inhibited ATPase of muscle, myosin, in the functioning of that tissue overshadows all work on ATPases. There exists also, however, in muscle, a labile ATPase which is activated by Mg^{++} and inhibited by Ca^{++} (Keilley & Meyerhof, 1948) and whose optimum pH, 6·8, is much closer to physiological pH than that of myosin, 9·4. Keilley & Meyerhof go so far as to suggest that *in vivo* the magnesium-activated ATPase must be responsible for most of the dephosphorylation which takes place in muscle. It does not seem to be very helpful to attempt at this stage to interpret the effects of Ca^{++} and Mg^{++} on nervous tissue through effects on ATPase. Low concentrations of both Ca^{++} and Mg^{++} in the blood produce hyperexcitability of the nervous system. On the other hand, anaesthesia produced by high Mg^{++} concentration in the blood is combated by calcium salts.

Meyerhof & Wilson (1949) have drawn some distinctions between soluble ATPase (that found in an extract of brain homogenate) and absorbed ATPase (that associated with the tissue particles of a homogenate). These workers also note different proportions of soluble and absorbed ATPase in brain, tumours and chicken embryo. It has been noticeable that in the preparation of brain homogenates in different solutions a fraction of the total ATPase is always found in the extract. This is the fraction of brain ATPase which has been treated by acetone precipitations by Binkley & Olson (1950) and which has yielded material active on both ATP and inorganic pyrophosphate. There would appear to be ATPase fractions in brain and other tissue which differ not only in solubility but possibly also in other properties. The problem of the isolation of these fractions in a pure state remains to be solved.

SUMMARY

1. A description is given of some of the properties of a magnesium-activated enzyme in guinea pig brain which splits two phosphate groups from adenosinetriphosphate.

2. The enzyme is inhibited by calcium ions, thiol group reactants and sodium fluoride. It is not inhibited by some barbiturate drugs and alkaloids, and only slightly by some structurally related substances.

3. The action of brain homogenates on adenosinetriphosphate has been compared with its action on inorganic pyrophosphate. While the conditions of action on the two substrates is similar in some respects, there is evidence that two separate enzymes are acting on the different substrates.

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Actions of Electrical Stimulation and of 2:4-Dinitrophenol on the Phosphates in Sections of Mammalian Brain *in vitro*

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The expectation that sections of brain *in vitro* might respond to electrical stimulation was based on the finding (McIlwain, Buchel & Cheshire, 1951) that resynthesis of labile phosphates could be induced in such preparations. When metabolic response to stimulation was found (McIlwain, 1951 and McIlwain, Anguiano & Cheshire, 1951) our working hypothesis was that the increase in respiration and glycolysis was secondary to breakdown of labile phosphates. The labile phosphates had presumably been depleted in supporting additional activity in the slices; their breakdown products stimulate both respiration and glycolysis (for references see McIlwain, 1950). The possible nature of the additional activity is discussed below.

An immediate test of such hypotheses was to examine the concentrations of inorganic and creatine phosphates in slices of brain stimulated electrically. Very little data are available on concentrations of phosphate fractions in surviving tissue slices during *in vitro* metabolism, but the value of such data in understanding the overall metabolism of nervous tissue has been shown by McIlwain, Buchel & Cheshire (1951) and Buchel & McIlwain (1950). In particular, we were also investigating in this laboratory the effects of substances such as 2:4-dinitrophenol which disturb the phosphorylation normally associated with respiration. We have therefore, in the present paper, compared the effects of electrical stimulation and of 2:4-dinitrophenol on phosphates, respiration and glycolysis.

EXPERIMENTAL

Guinea pig-brain cortex was used, and prepared as described by McIlwain (1951), where details of the salines are also given. The electrode vessels employed were type A^1 of Fig. 3 of that paper, with the tissue-holding electrodes *D* of Fig. 2, made of silver. Six vessels were commonly used together, of which up to four were stimulated. Each vessel held a slice of 100–120 mg., and two or three of these were combined for determination of phosphates as described by McIlwain, Buchel & Cheshire (1951). In previous determinations of phosphate after *in vitro* metabolism, dependable values were obtained so long as the slices were homogenized in trichloroacetic acid within about 30 sec. of their removal from oxygenated nutrient salines. To do this in the present experiments, the following procedure was adopted.

At the end of an experiment one stimulated vessel, with its manometer, was removed from the bath while its stimulating current was still maintained, the outside of the vessel quickly wiped dry around its joint with the manometer, and the manometer removed. The current was switched off, and the tissue-holding electrodes removed from the lead-in wires

while the electrodes and slice were still in the saline of the vessel. The tissue-holding electrodes and slice were removed with forceps and put into a shallow dish of the same oxygenated saline, the electrode opened, and the slice scooped out with a wire bent as shown in Fig. 1 *D* and mounted in a handle. When the electrode was opened the slice lay on its lower grid and the wire was of such a size and shape that it could be passed under the slice, between the wires of the lower grid and lifted out with the slice. The slice was put by means of the wire into an ice-cold test-tube homogenizer with trichloroacetic acid, operated by a second worker, and immediately disintegrated in the acid. The time from switching off the stimulating current to homogenizing was 30 sec. or less. The slice from a second manometric vessel was then treated in the same way. Commonly, to obtain sufficient tissue, more than one slice was extracted with the same trichloroacetic acid, but homogenizing of each slice followed promptly its removal from the vessel. The later slices thus metabolized 1–3 min. longer than the earlier, but this was known not to affect the phosphate levels.

Slices had been weighed before the beginning of the experiment, and in several cases the gross increase in weight on transferring them to the trichloroacetic acid at the end of the experiment was also obtained. This was done by weighing the homogenizer and its contents before the addition and after homogenizing. The increase in gross weight of the slices represented medium imbibed by or adhering to the slices. This was found to represent about 60 % of their initial wet weight. The inorganic phosphate of the medium was such that this represented an increase in the phosphates of about 0·5 μmol./g. tissue. This was small in comparison with the changes in inorganic phosphate described below, and moreover was common to stimulated and unstimulated tissues.

RESULTS

Electrical stimulation

Major changes in inorganic and creatine phosphates were found to follow electrical stimulation. This is shown in Tables 1 and 2. In performing these experiments it was necessary to find first whether the electrode arrangements and additional handling of the slices affected their phosphate contents. On the basis of previous experiments the grid type of tissue-holding electrode (Fig. 2 of McIlwain, 1951) appeared most suitable as the increase in respiration produced on stimulating in this electrode was maximal. Also the tissue was not appreciably damaged by these electrodes and could be released and collected relatively easily as is described in the experimental part. Five experiments with slices in these holders in glycylglycine-glucose salines gave mean values for inorganic phosphate of 3·30 and for creatine phosphate of 1·11 μmol./g. Values for free slices (McIlwain, Buchel & Cheshire, 1951) are 3·38 and 1·12 respectively. It can therefore be concluded that the holders and the procedure of handling the slices had no appreciable effect on the phosphates.

The experiments of Table 1 employed a.c. currents which increased respiration and glycolysis by 60–100 %. The effect on the phosphates was to lower considerably the creatine phosphate, and to raise the inorganic phosphate by about the same quantity or rather more. The ratio, inorganic P/creatine P, shown previously (Buchel & McIlwain, 1950) to be a sensitive index to changes in phosphates associated with metabolic changes, thus rises several-fold. It is instructive to compare these with changes caused by rise of temperature. As previously pointed out, an increase of the order of 0·05–0·2° may be caused by the currents used in stimulation. However, to increase respiration by 50 %, an increase in temperature of over 4° is required. Exp. 4, Table 1, shows that even this causes a very much smaller change in phosphates than is caused by electrical stimulation.

Effects of the currents were shown after brief periods of stimulation. Marked changes are seen in Exp. 3, Table 1, after 2·5 min. and in Exp. 3, Table 2, after 2 min.

Recovery from stimulation. It was found previously that when the stimulating current was stopped, respiration fell within a few minutes. Commonly,

Table 1. *Effects of electrical stimulation and temperature change on respiration, glycolysis and phosphates*

(Experiments were in glycylglycine-glucose saline with about 20 mg. tissue/ml. All slices of Exps. 1–3, stimulated and unstimulated, were in tissue-holding electrodes. Slices from three vessels, whose respiration was measured separately, were combined for determination of phosphates by Ca-ethanol separation. Portions of their salines were combined for determination of lactic acid. Currents (sine-wave a.c.) were applied in Exps. 1–3 about 5 min. after the vessels were put at 37°.)

Exp. no.	Temp. (°)	Voltage applied	Duration (min.) of: Experiment	Duration (min.) of: Stimulation	Respiration (μmol./g./hr.)	Lactic acid (μmol./g.)	Inorganic phosphate (μmol./g.)	Creatine phosphate (μmol./g.)	Ratio $\frac{\text{inorganic P}}{\text{creatine P}}$
1	37	0	40	—	56, 64, 54	41	4·0	1·18	3·5
1	37	2·7	40	35	104, 96, 98	61	5·0	0·27	18·1
2	37	0	30	—	75, 69, 69	43	3·03	1·10	2·75
2	37	2·9	30	25	138, 141, 153	79	4·54	0·51	8·9
3	37	0	32	—	—	—	4·1	1·10	3·6
3	37	2·8	32	2·5	—	—	5·3	0·64	8·6
4	37	0	60	—	56, 56, 63	37	3·0	1·19	2·5
4	41	0	60	—	75, 75, 73	50	3·8	1·00	3·8

Table 2. *Recovery from effects of electrical stimulation*

(Experimental arrangements: as Table 1 except where indicated otherwise. Most of the periods of metabolism were too brief to obtain accurate measurements of the rate of respiration, but where observable, stimulation was found to lead to a marked increase of the order of that recorded in Table 1.)

Exp. no.	Period (min.)	Voltage	Lactic acid (μmol./g.)	Inorganic phosphate (μmol./g.)	Creatine phosphate (μmol./g.)
1*a*	0–40	2·9	56	3·48	1·35
1*b*	0– 5	0	125	5·4	0·57
	5–40	2·9			
1*c*	0– 5	0	115	4·3	0·78
	5–25	2·9			
	25–40	0			
2*a*	0–65	0	47	4·3	1·25
2*b*	0–25	0	57·1	5·5	0·61
	25–45	2·7			
2*c*	0–25	0	67·2	4·7	1·00
	25–50	2·7			
	50–65	0			
3*a*	0–43	0	—	3·5	1·51
3*b*	0–21	0	—	5·4	0·87
	21–23	2·8			
3*c*	0–21	0	—	3·7	1·22
	21–23	2·8			
	23–43	0			

Table 3. *Concentration and action of* 2:4-*dinitrophenol*

(All salines contained glucose, and the dinitrophenol where indicated was present before the tissue was added. Exp. 3 employed Warburg's indirect method for gaseous exchange. Experiments were at 37° for 60–90 min., during which time respiration was linear.)

Exp. no.	Medium	Dinitrophenol (M)	Respiration (μmol./g./hr.)	Lactic acid (μmol./g./hr.)
1	Phosphate	0	60	22
1	Phosphate	9×10^{-6}	93	35
1	Phosphate	$4{\cdot}5 \times 10^{-5}$	139	49
1	Phosphate	$2{\cdot}3 \times 10^{-4}$	24	86
2	Phosphate	0	67	19
2	Phosphate	5×10^{-5}	121	37
3	Bicarbonate	0	62	42
3	Bicarbonate	$2{\cdot}1 \times 10^{-5}$	—	55
3	Bicarbonate	5×10^{-5}	120	73
4	Glycylglycine	0	60	30
4	Glycylglycine	2×10^{-5}	115	44
4	Glycylglycine	5×10^{-5}	125	52
4	Glycylglycine	1×10^{-4}	95	73
4	Glycylglycine	2×10^{-4}	69	97

Table 4. 2:4-*Dinitrophenol on phosphates*

(Glycylglycine-glucose saline was employed at 37°, with about 30 mg. tissue/ml. Phosphates in Exps. 1 and 2 were by differential stability and in Exp. 3 by Ca-ethanol separation.)

Exp. no.	Period of metabolism (min.)	Period of contact with 2:4-dinitrophenol (min.)	Dinitrophenol (M)	Inorganic phosphate (μmol./g.)	Creatine phosphate (μmol./g.)
1*a*	75	—	0	3·8	1·19
1*b*	75	75	10^{-5}	5·0	0·29
2*a*	75	—	0	3·65	1·51
2*b*	75	75	10^{-4}	5·30	0·58
3*a*	45	—	0	3·0	1·0
3*b*	45	0·5–2	5×10^{-5}	5·5	0·22
3*c*	45	0·5–2	5×10^{-5}	4·7	0·50

rates approximately equal to those before stimulation were reached in 5 min., but sometimes a higher rate persisted. Phosphates have been found to show a partial return to normal within 15 min., which was not, however, complete in 25 min. In Exp. 1, Table 2, stimulation was commenced after only 5 min. at 37°. The creatine phosphate of freshly cut slices is low and may not, therefore, have been completely resynthesized before stimulation was applied. In Exp. 2 a period of 25 min. is allowed for the initial resynthesis and this period is known to be fully adequate. Stimulation then halved the values for phosphates, and in a 15 min. recovery period a large part of the loss has been made good. In each case inorganic phosphate was increased by stimulation and lowered in the period of normal metabolism following stimulation. Very similar changes are seen to follow a brief period of stimulation, in Exp. 3.

2:4-*Dinitrophenol*

The action of dinitrophenol on glucose metabolism by guinea pig-brain cortex was found to be similar to that recorded in other systems, and included (Table 3) large increases in respiration and aerobic glycolysis. Respiration was also inhibited by the higher concentrations of the drug. The change in phosphates was studied (Table 4) at concentrations of dinitrophenol which increased respiration and glycolysis. Large changes in phosphates were readily observed. In Exps. 1 and 2 of Table 4 the tissues treated with 2:4-dinitrophenol were in contact with it during the whole of its period of metabolism. This led to concentrations of creatine phosphate which were one-third or less of those in slices which had been in the same medium but without the drug. Inorganic phosphate rose considerably.

The time needed for these changes was found to be remarkably short. In Exp. 3, Table 4, an ample preliminary period of metabolism had been allowed for resynthesis of creatine phosphate. 2:4-Dinitrophenol to form a solution of only 5×10^{-5} M was then added, and the slices removed and put into trichloroacetic acid at times between 0·5 and 2 min. after the addition. This short time sufficed for the drug to penetrate the slices (some 0·35 mm. thick) and greatly reduce the creatine phosphate. Inorganic phosphate again was raised.

Joint action of 2:4-*dinitrophenol and electrical stimulation*

The largest changes observed to be caused in respiration by the dinitrophenol and by applied potential gradients are similar in magnitude. So also are the changes in phosphates. The loss in creatine phosphate in the two cases cannot be entirely cumulative as each may cause the loss of the greater part of the creatine phosphate. It is therefore relevant to understanding the actions of the two agents, to see whether their effects on respiration are additive.

Fig. 1 shows that the respiratory effects are not additive. Here the initial effect of the applied potential gradient was to double the rate of respiration; later the rate fell off, but it remained much higher than the initial rate. 2:4-Dinitrophenol also approximately doubled respiration, and application of the same potential then had only a very small further effect. Other experiments showed that the effects on glycolysis, also, were additive to only a limited extent.

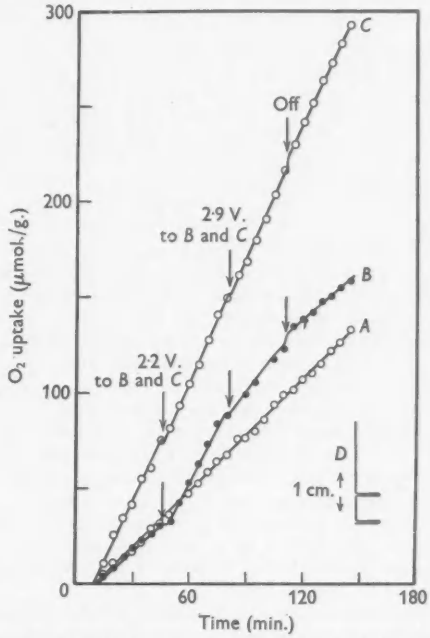

Fig. 1. *A* to *C*, effects of dinitrophenol and of electrical stimulation on respiration of guinea pig-brain cortex slices. *D*, wire used for lifting slices from tissue-holding electrodes. The respiration was measured in glucose-phosphate saline at 37° and the slices were in tissue-holding electrodes type *D* in vessels *A*[1] (McIlwain, 1951). Slice *A* was unstimulated; to slices *B* and *C*, sine-wave a.c. was applied as indicated. The medium in the case of slice *C* contained 5×10^{-5} M-2:4-dinitrophenol.

DISCUSSION

Remarkably close similarity in biochemical changes has now been found between the effects of electrical stimulation on the cerebral cortex of intact animals, and on slices of the cortex in the newly devised experimental conditions described above. The changes measured include respiration, lactic acid formation, glucose utilization, and inorganic phosphate

formation which all increase, and creatine phosphate level which decreases. Reasons have been given (McIlwain, 1950) for considering the changes on stimulation *in vivo* to represent a coordinated series initiated by utilization of labile phosphates in supporting increased electrical activity in the cortex. Inasmuch as depletion of the labile phosphates during *in vitro* stimulation was anticipated on the basis of such conceptions, this finding can be regarded as supporting the interconnexion suggested. Moreover, the same set of changes is brought about by a very different type of agent, 2:4-dinitrophenol. The effects of this substance are known, from experiments in cell-free systems and with substances simpler than glucose as substrates, to be primarily on phosphorylation (see, for example, in the case of brain cortex, Case & McIlwain, 1951). In the present experiments the dinitrophenol can thus be regarded as leaving available for glucose catabolism a higher concentration than normal of inorganic phosphate and phosphate acceptors. At this point it could therefore influence respiration and glycolysis in the same fashion as electrical stimulation. Other interrelations between the metabolic changes observed do not appear reasonable, e.g. that increased glucose catabolism should decrease labile phosphates and increase inorganic phosphate. Examples of increase in respiration and in relevant anaerobic reactions of brain preparations caused by concentrations of phosphate and phosphate acceptors involved in the present experiments have been quoted previously (Banga, Ochoa & Peters, 1939; Long, 1945; McIlwain, Buchel & Cheshire, 1951).

The experiments with 2:4-dinitrophenol show that if one were to judge only by these metabolic effects, the actions of applied potential gradients could be due to their inhibiting phosphorylation rather than increasing the utilization of the labile phosphates formed. Experiments by different techniques are needed to decide this matter, but the similarity previously remarked (McIlwain, 1951) between the potential gradients effective *in vivo* and *in vitro* count in favour of their acting in our experiments as they do *in vivo*, i.e. by causing increased utilization of labile phosphates. Similar conclusions were arrived at with respect to peripheral nerve some time ago (Gerard, 1932).

It is also possible that any increased utilization of phosphates in our experiments might not be due to increased functional activity of the tissue. The potent adenosinetriphosphatase of brain cortex (see Gore, 1951) is noteworthy and the electrical energy applied in the present experiments might in some way facilitate its contact with its substrate without causing any increased electrical activity in the slice. However, by choosing impulses of suitable frequency and duration, much more electrical energy than that of the present experiments can be expended at a slice in a given time, without causing stimulation of respiration (unpublished observations). The balance of present evidence thus favours the suggestion that the changes observed *in vitro* bear a close relation to those observed *in vivo*. They emphasize that the increased metabolism of the brain on electrical stimulation in the whole animal need not be secondary to the vasomotor or endocrine changes which are observed to accompany it.

SUMMARY

1. Procedures have been developed for sampling, within half a minute, sections of cerebral cortical tissue to which potential gradients have been applied during *in vitro* metabolism.

2. The potential gradients which caused increased respiration and glycolysis in sections of mammalian brain caused also a fall in their content of creatine phosphate and a rise in that of inorganic phosphate.

3. The changes in phosphates could be induced by stimulation at different times during the *in vitro* metabolism. By stopping the stimulation and maintaining good metabolic conditions for about 15 min. the inorganic and creatine phosphates were partly restored to their previous, unstimulated values.

4. The increase in respiration caused by 2:4-dinitrophenol was maximal at about a 5×10^{-5} M concentration (approx. 20 mg. tissue/ml. fluid). The maximum value was appropriately equal to the maximum respiration caused by electrical stimulation. The two effects, when each was approaching its maximum, were not additive; potential gradients normally stimulatory had negligible effects when applied to a slice metabolizing in the presence of 5×10^{-5} M-dinitrophenol.

5. 2:4-Dinitrophenol also caused a fall in the creatine phosphate and a rise in the inorganic phosphate of brain-cortex slices. It was very prompt in doing this, having a large effect within 2 min. of its addition.

We are greatly indebted to Mr J. Cheshire and Miss S. Russell for assistance during these experiments.

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The Action of Peroxidase Systems on Ferrocyanide, Molybdate, Tungstate and Vanadate

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Kenten & Mann (1950) showed that plant peroxidase preparations in the presence of certain phenolic substrates catalyse the oxidation of Mn^{++} by hydrogen peroxide. Evidence was obtained that the Mn^{++} reduces an intermediate oxidation product of the phenolic substrate and thereby is itself oxidized. The oxidizing capabilities of the system peroxidase + phenolic substrate + hydrogen peroxide towards inorganic compounds have now been further investigated. It is shown that the system oxidizes ferrocyanide, and evidence suggesting the oxidation of molybdate, tungstate and vanadate is reported.

MATERIALS AND METHODS

Peroxidase preparations were obtained from horse-radish by the method of Keilin & Mann (1937). The 'Purpurogallinzahl' (P.Z.) of these preparations was determined by the method of Keilin & Mann (1937).

Hydrogen peroxide. In the experiments with molybdate, tungstate and vanadate, approximately 0·05 M-H_2O_2 was used, and for the manometric experiments with ferrocyanide approximately 0·025 M-H_2O_2 in 0·0105 M-$NaHCO_3$. Fresh solutions were prepared for each experiment. The 0·025 M-H_2O_2 in $NaHCO_3$ was estimated by $KMnO_4$ titration and the 0·05 M-H_2O_2 manometrically with MnO_2 in acid solution (Kenten & Mann, 1950).

Ammonium permolybdate was prepared by treatment of 100 vol. H_2O_2 with molybdate according to the method of Rosenheim, Hakki & Krause (1932). The peroxidic oxygen was estimated by thiosulphate titration of the I_2 liberated from KI in acid solution by the permolybdate. Values obtained in various preparations varied from 7·8 to 9·6%. Rosenheim *et al.* (1932) found 7·1–7·5% peroxidic oxygen.

Uranium peroxide was prepared by treatment of uranyl nitrate with H_2O_2 at 40–50° according to the method of Watt, Achorn & Marley (1950). Peroxidic oxygen varied from 9·2 to 9·4%. Watt *et al.* (1950) found 9·0%.

Manometric measurements were carried out in the Warburg apparatus at 25°. The experiments with molybdate, tungstate and vanadate were made in phosphate buffers; the volume of the reaction mixture was 3 ml., KOH was present in the centre cup and the gas space was air. The ferrocyanide experiments were made in bicarbonate media; the volume of the reaction mixture was 4·5 ml., KOH was omitted from the centre cup and the gas space was 95% N_2 + 5% CO_2.

EXPERIMENTAL AND RESULTS

Oxidation of ferro- to ferricyanide by peroxidase systems

Colorimetric evidence. Reaction mixtures consisting of horse-radish peroxidase of P.Z. 105 (40 μg. in 0·5 ml. water), 1 ml. 0·025 M-$K_4Fe(CN)_6$, 1 ml. M-pyrophosphate at pH 7 and 0·3 ml. 0·1 M-phenolic substrate were made up in test tubes and 0·3 ml. 0·05 M-H_2O_2 was added. Control experiments were made in which each of the reactants was omitted in turn, or in which both phenolic substrate and peroxidase were omitted. With the complete system, using either phenol, *p*-cresol or *o*-cresol, the reaction mixture within 1 min. developed a strong yellow colour indicating ferricyanide formation. With peroxidase heated for 15 min. in a boiling-water bath or with H_2O_2 in absence of peroxidase little or no oxidation of ferrocyanide could be observed within this time, but with peroxidase in absence of phenolic substrate a faint yellow colour had developed in 1 min. The results suggested that peroxidase alone catalyses the oxidation of ferro- to ferri-cyanide by H_2O_2, but that the system formed by peroxidase, together with a suitable phenolic substrate, was a much more active catalyst of the reaction.

Manometric evidence. The oxidation of ferro- to ferri-cyanide is accompanied by the consumption of H^+, and may therefore be followed manometrically by measurement of CO_2 uptake in bicarbonate media in an atmosphere of $N_2 + CO_2$ (cf. Haas, 1937; Quastel & Wheatley, 1938).

$$H_2O_2 + 2Fe(CN)_6^{----} + 2H^+ \rightarrow 2Fe(CN)_6^{---} + 2H_2O, \quad (1)$$

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-. \quad (2)$$

Equations 1 and 2 show that for each molecule of H_2O_2 used in the oxidation of ferrocyanide two molecules of CO_2 will be taken up.

Reaction mixtures consisted of 3 μg. peroxidase of P.Z. 480, 0·6 ml. of 0·05 M-$K_4Fe(CN)_6$, 0·45 ml. of 0·1 M-*p*-cresol in 0·0105 M-$NaHCO_3$; 0·2 ml. of 0·025 M-H_2O_2 in 0·0105 M-$NaHCO_3$ was added from the side arm. The total volume of the reaction mixtures was 4·5 ml. The gas space was filled with 95% N_2 + 5% CO_2. Under these conditions the reaction of the medium after equilibration was about pH 7. Control experiments were made in which either *p*-cresol or peroxidase was omitted.

A typical set of results is shown in Fig. 1. On the addition of the H_2O_2 from the side arm a rapid uptake of CO_2 took place, where both peroxidase and *p*-cresol were present. The observed uptake, which was complete in about 10 min., varied in different experiments between 90 and 110% of theory based on the H_2O_2 added. In the control experiments in absence

of peroxidase the initial rate of uptake was about 15 % and in absence of *p*-cresol about 30 % of the rate where the complete system was present. The results show that peroxidase in presence of *p*-cresol catalyses the oxidation of ferrocyanide by H_2O_2. In further experiments it was found that phenol could be substituted for *p*-cresol, but negative results were obtained with pyrogallol and catechol. Similarly, Kenten & Mann (1950) found that the oxidation of Mn^{++} by peroxidase systems could be demonstrated with phenol or *p*-cresol, but not with pyrogallol or catechol as phenolic substrates.

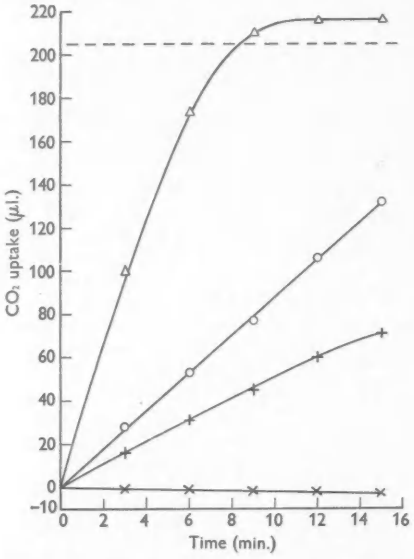

Fig. 1. The effect of peroxidase and *p*-cresol on the oxidation of ferrocyanide by H_2O_2. △—△, peroxidase + *p*-cresol + ferrocyanide; ○—○, peroxidase + ferrocyanide; +—+, ferrocyanide; ×—×, peroxidase + *p*-cresol. H_2O_2 added to all reaction mixtures. The dotted line shows the theoretical uptake.

The results of Fig. 1 also suggest that peroxidase in absence of *p*-cresol may catalyse the oxidation of ferrocyanide by H_2O_2 though at a much slower rate than when *p*-cresol also is present. This was shown more clearly by using larger amounts of peroxidase (Fig. 2). The reaction mixtures were made up as before except that *p*-cresol was omitted and the peroxidase additions varied between 0 and 32 μg. of P.Z. 480. A linear relationship was found between the increased rate of CO_2 uptake and peroxidase added up to 16 μg. of P.Z. 480. The rate could be still further increased with larger amounts of peroxidase, though here the relationship was not linear. Control experiments showed that peroxidase, which had been heated for 15 min. in a boiling-water bath, had no effect on the rate of CO_2 uptake. Comparison of Figs. 1 and 2 shows that 3 μg. of the peroxidase preparation in presence of *p*-cresol catalyses the oxidation of ferrocyanide by H_2O_2 at about the same rate as 16 μg. of the peroxidase preparation in absence of *d*-cresol.

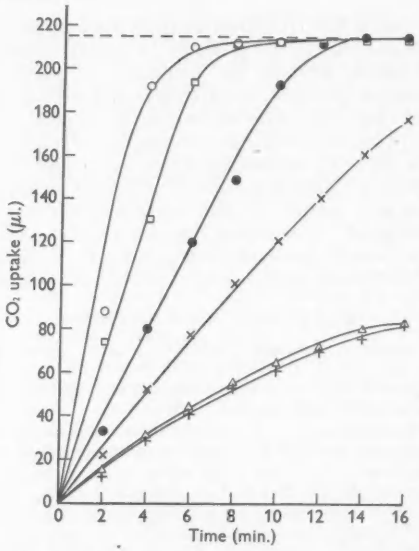

Fig. 2. The effect of peroxidase on the oxidation of ferrocyanide by H_2O_2. Peroxidase added, ○—○, 32 μg.; □—□, 16 μg.; ●—●, 8 μg.; ×—×, 4 μg.; △—△, 0 μg.; +—+, 32 μg., heated for 15 min. at 100°. The dotted line shows the theoretical uptake.

Oxidation of molybdate, vanadate and tungstate

Effects of molybdates, tungstates, and vanadates on the oxidation of Mn^{++} *by peroxidase systems.* It has previously been shown (Kenten & Mann, 1949, 1950) that the oxidation of manganese by peroxidase systems can be followed manometrically by measuring the evolution of O_2 produced when the manganese oxidation product reacts stoicheiometrically with the H_2O_2. Assuming the manganese oxidation product is Mn_2O_3, the reactions can be represented by the equations:

$$\text{Phenolic substrate} + H_2O_2 \xrightarrow{\text{Peroxidase}} \text{oxidized phenolic substrate}, \quad (3)$$

$$\text{Oxidized phenolic substrate} + MnO \rightarrow \text{phenolic substrate} + Mn_2O_3, \quad (4)$$

$$Mn_2O_3 + H_2O_2 \rightarrow MnO + O_2 + H_2O. \quad (5)$$

Under suitable conditions complete decomposition of added H_2O_2 is observed. If insufficient Mn^{++} is

present to reduce the oxidized phenolic substrate, this is further oxidized to products which Mn^{++} cannot reduce. When Mn^{++} is the limiting factor the evolution of O_2 rapidly ceases owing to the complete loss by irreversible oxidation of the phenolic substrate. In these circumstance the addition of small amounts of molybdates, tungstates or vanadates markedly increased both the rate of evolution and the total O_2 output. Results illustrating this effect with ammonium molybdate are shown in Fig. 3.

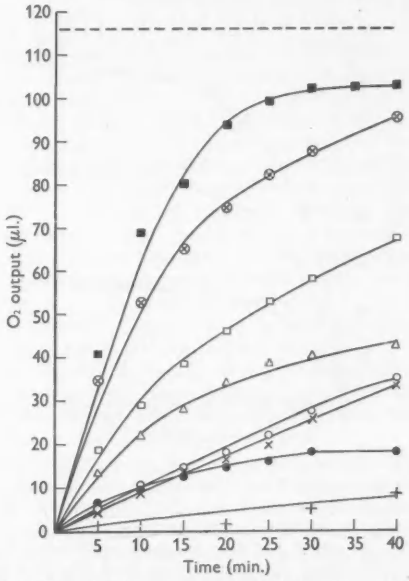

Fig. 3. The effect of ammonium molybdate on the oxidation of Mn^{++} by the system peroxidase, *p*-cresol and H_2O_2. Peroxidase (2 μg.) + *p*-cresol + Mn^{++} (5·5 μg.) with the following concentrations of ammonium molybdate: ■—■, $9{\cdot}6 \times 10^{-6}$ M; ⊗—⊗, $4{\cdot}8 \times 10^{-6}$ M; □—□, $2{\cdot}4 \times 10^{-6}$ M; △—△, $1{\cdot}2 \times 10^{-6}$ M; ●—●, 0. Controls with $9{\cdot}6 \times 10^{-6}$ M-ammonium molybdate; ○—○, *p*-cresol omitted; ×—×, peroxidase omitted; +—+, Mn^{++} omitted. The dotted line shows the theoretical output.

Reaction mixtures consisted of 2 μg. peroxidase of P.Z. 250, 0·2 ml. of 0·005 M-*p*-cresol, 0·1 ml. 0·001 M-$MnSO_4$ (5·5 μg. Mn^{++}), and varying amounts of ammonium molybdate (2·4–192 μg. Mo) in 0·033 M-orthophosphate at pH 7; 0·2 ml. of 0·05 M-H_2O_2 was added from the side arm after equilibration. Control reaction mixtures were also made in which either $MnSO_4$, ammonium molybdate or *p*-cresol were omitted. In absence of molybdate the total O_2 output was about 20 μl.; where molybdate was also present, both the rate and total O_2 output were markedly increased even at the lowest molybdate concentration of $1{\cdot}2 \times 10^{-6}$ M (2·4 μg. Mo). With the highest molybdate concentration ($9{\cdot}6 \times 10^{-6}$ M) the O_2 output was complete in about 30 min. and was equivalent to decomposition of about 90% of the H_2O_2 added. Where $MnSO_4$ was omitted from the reaction mixtures the decomposition of H_2O_2 produced by molybdate at concentrations of 10^{-5} to 10^{-6} M was negligible. The decomposition of H_2O_2 observed in the presence of both $MnSO_4$ and molybdate was therefore not merely a summation of the separate effects of molybdate and $MnSO_4$. Similar effects were obtained with tungstates and vanadates in place of molybdates but not with borate, chromate and uranate.

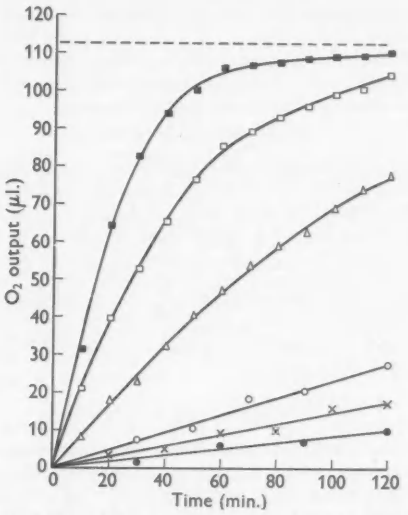

Fig. 4. The effect of ammonium molybdate on the oxidation of Mn^{++} by H_2O_2. Mn^{++} (5·5 μg.) with the following concentrations of ammonium molybdate: ■—■, $7{\cdot}5 \times 10^{-5}$ M; □—□, $3{\cdot}7 \times 10^{-5}$ M; △—△, $9{\cdot}6 \times 10^{-6}$ M; ○—○, $1{\cdot}2 \times 10^{-6}$ M; ●—●, 0. ×—×, control with $7{\cdot}5 \times 10^{-5}$ M-ammonium molybdate, Mn^{++} omitted. The dotted line represents the theoretical output.

The oxidation of Mn^{++} *by* H_2O_2 *in presence of molybdates, tungstates and vanadates*

In the absence of *p*-cresol or peroxidase, $MnSO_4$ and molybdate together, at the concentrations used in the previous experiment, caused a decomposition of H_2O_2 which was slow in comparison with that produced when the enzyme and its substrate were also present, but much greater than the sum of the effects of $MnSO_4$ and molybdate separately. The rate of this decomposition of H_2O_2 by $MnSO_4$ and molybdate in absence of peroxidase and *p*-cresol could be increased by increasing the concentration of molybdate (Fig. 4).

Reaction mixtures consisted of 0·1 ml. 0·001 M-$MnSO_4$ (5·5 μg. Mn^{++}) and varying amounts of ammonium molybdate (2·4–150 μg. Mo) in 0·033 M-orthophosphate at pH 7; 0·2 ml. 0·05 M-H_2O_2 was added from the side arm after equilibration. The results suggest that if the concentration of molybdate is increased to a sufficiently high level, the rate of decomposition of H_2O_2 may be as rapid as that obtained with small amounts of molybdate in the presence of peroxidase and *p*-cresol. But at the highest concentration of molybdate used ($7·5 \times 10^{-5}$ M) the rate was about half that obtained at one-eighth the concentration when peroxidase and *p*-cresol were also present (Fig. 1). Similar results were obtained using tungstates or vanadates in place of molybdates, but not with borate, chromate or uranate.

It is known that H_2O_2 oxidizes molybdates, tungstates, vanadates, chromates and uranates to peracids according to the general equation

$$X.OH + H_2O_2 \rightleftharpoons X.O.OH + H_2O. \quad (6)$$

The oxidation takes place readily at room temperature. The experiments in the next section show that permolybdates oxidize Mn^{++} and suggest that the interaction between molybdates and Mn^{++} in the decomposition of H_2O_2 is due to the formation of permolybdates. Since peroxidase and *p*-cresol catalyse the molybdate effect at low concentrations of molybdate it is suggested that peroxidase and *p*-cresol catalyse the formation of permolybdates under these conditions.

Oxidation of Mn^{++} *by permolybdates*

Manometric evidence. MnO_2 and manganipyrophosphate rapidly decompose aqueous solutions of permolybdate with the evolution of O_2. If permolybdate oxidizes Mn^{++}, O_2 should be evolved as a result of the reaction and under conditions where the oxidized manganese does not accumulate the O_2 output is a measure of the Mn^{++} oxidized. Assuming the oxidation is to the manganic state the reaction can be represented as follows:

$$X.O.OH + 2MnO \rightarrow X.OH + Mn_2O_3, \quad (7)$$

$$Mn_2O_3 + X.O.OH \rightarrow 2MnO + O_2 + X.OH. \quad (8)$$

This was tested in reaction mixtures consisting of 0·1 ml. 0·001 M-$MnSO_4$ in 0·033 M-orthophosphate at pH 7; varying amounts of a solution of ammonium permolybdate were added from the side arm after equilibration.

In the presence of $MnSO_4$ a rapid evolution of O_2 took place, the total O_2 output in each case being approximately equivalent to the peroxidic oxygen in the permolybdate (Fig. 5). This is consistent with the hypothesis that permolybdate oxidizes Mn^{++}.

Isolation of MnO_2. Kenten & Mann (1949), while studying the oxidation of Mn^{++} by peroxidase systems, found that in pyrophosphate solutions at high concentrations of Mn^{++} the rate of oxidation of Mn^{++} was greater than its rate of reduction by the H_2O_2, and manganipyrophosphate therefore accumulated. By the addition of alkali the manganipyrophosphate was converted to MnO_2 which was isolated. Under similar conditions it has been possible to isolate small amounts of MnO_2 from large-scale reaction mixtures of $MnSO_4$ and ammonium permolybdate, thus obtaining direct proof that permolybdate oxidizes Mn^{++}.

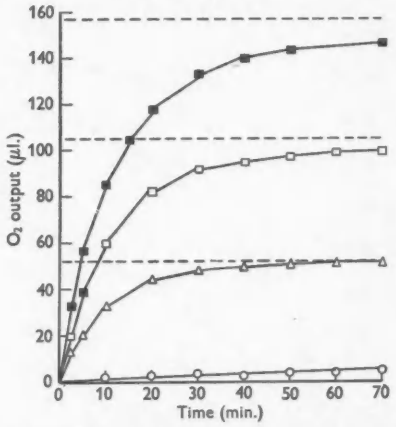

Fig. 5. The effect of Mn^{++} on the decomposition of ammonium permolybdate. Mn^{++} (5·5 μg.) with the following amounts of ammonium permolybdate (peroxidic oxygen 9·4%): ■—■, 4·8 mg.; □—□, 3·2 mg.; △—△, 1·6 mg.; ○—○, control with 4·8 mg. ammonium permolybdate, Mn^{++} omitted. The dotted lines show the theoretical output.

To a reaction mixture containing 40 ml. of 0·5 M-$MnSO_4$ and 300 ml. of M-pyrophosphate at pH 7, 1·6 g. of ammonium permolybdate (8·2% peroxidic oxygen) in 8 ml. of water were added. The colour of the reaction mixture which was initially pale yellow turned through orange to deep red within 5 min. The reaction mixture was allowed to stand for 4 hr. and a white precipitate which had formed was then centrifuged off. The supernatant was adjusted to pH 10 (glass electrode) and the MnO_2 which precipitated was isolated and analysed by the procedure of Kenten & Mann (1949). The yield of MnO_2 (62 mg.; valency 4·03) was 17% based on the permolybdate added.

DISCUSSION

The results of the present work show that peroxidase in presence of suitable phenolic substrates catalyses the oxidation of ferrocyanide by hydrogen peroxide and possibly that of molybdates, vanadates and

tungstates, though in the latter cases direct proof of the oxidation was not obtained. Molybdate, vanadate and tungstate catalyse the oxidation of Mn^{++} by peroxidase systems, and it is inferred that this catalysis is brought about through the oxidation of these complex ions to peracids.

According to Jahr (1941) the oxidation of sodium molybdate by hydrogen peroxide proceeds according to the following equations:

$$MoO_4^{--} + 2H_2O_2 \rightleftharpoons HMo_6^- + H_2O + OH^-, \quad (9)$$

$$MoO_4^{--} + 4H_2O_2 \rightleftharpoons MoO_8^{--} + 2H_2O. \quad (10)$$

In the present work, in agreement with eqn. 9, a carbon dioxide uptake was found when hydrogen peroxide was added to sodium molybdate in bicarbonate buffer at pH 7 in presence of nitrogen-carbon dioxide. Peroxidase and *p*-cresol had little effect on the rate of this uptake. In such experiments a relatively high concentration of sodium molybdate, e.g. 10^{-2} to 10^{-3} M, has to be employed to obtain a reasonable carbon dioxide uptake, whereas the indirect evidence that peroxidase catalyses permolybdate formation was obtained at concentrations of 10^{-5} to 10^{-6} M-ammonium molybdate.

In absence of phenolic substrate peroxidase catalyses the oxidation of ferrocyanide by the hydrogen peroxide, though at a much reduced rate, but no evidence has been found that peroxidase alone catalyses the oxidation of molybdates, vanadates and tungstates by hydrogen peroxide.

These results, in conjunction with those previously obtained on manganese oxidation (Kenten & Mann, 1950), are of possible significance in considering the functions both of peroxidase and of certain micronutrients in plant metabolism. It has been suggested that tyrosinase may act as a terminal oxidase in plant respiration, the quinone formed by the action of the oxidase acting as a hydrogen acceptor (e.g. Boswell & Whiting, 1938; Baker & Nelson, 1943). Since quinones oxidize coenzyme I (Dixon & Zerfas, 1940) and coenzyme II (Kubowitz, 1937), tyrosinase could thus act as a link between oxygen and dehydrogenases requiring these coenzymes. In a similar manner peroxidase could link enzymes producing hydrogen peroxide to such dehydrogenase systems. It is possible that manganese, molybdates, vanadates and tungstates can form a link between peroxidase and plant metabolites and dehydrogenase systems other than those dependent on coenzymes I and II, and thus extend the range of peroxidase activity. Chance (1949) has shown that peroxidase oxidizes ferrocytochrome *c*, which suggests a possible connexion between peroxidase and plant respiration dependent on carriers containing ferrous iron as the active group. The results of the present work suggest that such carriers need not necessarily be haemoproteins. In this connexion it is of interest that laccase catalyses the oxidation of ferrocyanide by oxygen (Gregg & Miller, 1940).

The available evidence indicates that the main biological importance of molybdenum in plant metabolism is in connexion with the nitrogen cycle, e.g. in the fixation of atmospheric nitrogen by the free-living organisms *Azotobacter* and *Clostridia*, and the root nodule bacteria *Rhizobia* (Bortels, 1930; Horner, Burk, Allison & Sherman, 1942), and in the process of nitrate assimilation (Hewitt & Jones, 1947; Hewitt, Jones & Williams, 1949; Mulder, 1948). It is possible, however, that the biological activity of molybdenum is not restricted to its effect on nitrogen metabolism and that its effect on nitrate assimilation and nitrogen fixation is not directly on the reduction processes involved, but on the associated oxidative reactions which supply the energy required by these processes. Hewitt, Agarwala & Jones (1950) find that molybdenum deficiency consistently causes a striking reduction in the apparent ascorbic acid content of plants, and discuss the possibility that ascorbic acid may function in plants as an agent in nitrate reduction.

The oxidation of iron, manganese and molybdate in plants *in vivo* by peroxidase systems depends on the formation of hydrogen peroxide by the plant tissue. Work in progress shows that manganese is oxidized by plant extracts and that such oxidation depends on the formation of hydrogen peroxide by the extracts. In fact, this oxidation of manganese forms a useful test for the presence of enzyme systems producing hydrogen peroxide.

SUMMARY

1. Plant peroxidase catalyses the oxidation of ferrocyanide by hydrogen peroxide. The system formed by peroxidase, together with a suitable phenolic substrate, is a much more effective catalyst of the reaction.

2. Under certain conditions the rate of oxidation of manganese by peroxidase systems is markedly increased by adding molybdates, tungstates or vanadates.

3. Evidence has been obtained that this effect is due to catalysis by peroxidase systems of the oxidation of molybdates, tungstates and vanadates to the corresponding peroxy compounds by hydrogen peroxide.

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The Assimilation of Amino-Acids by Bacteria

13. THE EFFECT OF CERTAIN AMINO-ACIDS ON THE ACCUMULATION OF FREE GLUTAMIC ACID BY *STAPHYLOCOCCUS AUREUS*: EXTRACELLULAR PEPTIDE FORMATION

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If washed suspensions of *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) are incubated in buffered salt solution containing glutamic acid and glucose, the amino-acid enters and accumulates in the free state within the cells (Gale, 1947). The passage of glutamic acid into the cells is energy-linked and can be uncoupled from glycolysis by inhibitors such as sodium azide, 2:4-dinitrophenol, aureomycin and 8-hydroxyquinoline (Gale, 1949, 1951*a*; Gale & Paine, 1951). If a complete mixture of natural amino-acids is added to the external medium, the accumulation of free glutamic acid within the cells ceases, and glutamic acid becomes incorporated within the cellular material in a combined form from which it can be liberated by acid hydrolysis (Gale, 1951*b*). The addition of incomplete mixtures of amino-acids to the external medium results in a reduction in the rate of accumulation of free glutamic acid within the cells (Gale, 1951*b*), and the present communication deals with the mechanism of this inhibition.

EXPERIMENTAL

Organism. The organism used throughout this work was *Staphylococcus aureus* Duncan (Gale & Taylor, 1947).

Conditions of growth. The organism was grown for 16 hr. at 30° in the 'deficient medium B' consisting of a salt-mixture solution containing 1% glucose, 0·1% arginine and 0·2% marmite (Gale, 1947). The medium was distributed in 150 ml. quantities in Roux bottles.

Determination of glutamic and aspartic acids. Glutamic acid was determined manometrically by use of the specific glutamic acid decarboxylase preparation (Gale, 1945). Aspartic acid was determined by the manometric method of Krebs (1950) using glutamic acid decarboxylase in conjunction with glutamic-aspartic transaminase. The accumulation of these acids within the cells was studied by the methods previously described (Gale, 1947, 1951*b*).

Determination of combined glutamic acid. For the determination of total glutamic acid, thick suspensions of cells were hydrolysed for 18 hr. in boiling 6N-HCl, the acid removed by evaporation to dryness *in vacuo*, the hydrolysate dissolved in water and the pH adjusted to 4·5 before estimation with the decarboxylase preparation. The total glutamic acid was then corrected for the free glutamic acid content to give combined glutamate. Solutions obtained after removal of cells by centrifuging were also examined for combined glutamate in a similar fashion.

Chromatographic investigation of solutions. Incubation mixtures were freed from bacteria by centrifuging and then reduced to one-tenth of their original volume by evaporation under reduced pressure at a temperature below 40°; after neutralization they were deproteinized by the addition of 1 vol. ethanol and 6 vol. $CHCl_3$, the samples centrifuged and the aqueous layer used for chromatographic examination.

The general procedures used for paper chromatography were those described by Consden, Gordon & Martin (1944).

Whatman filter papers nos. 1 or 4 were used, except for the separation of larger amounts of material or to increase the concentration of components present in small amount in which case Whatman paper no. 3 was used. Two solvents were used: butanol-acetic acid-water mixture (Woiwod, 1949) and a propanol-ammonia (0·3%) mixture containing 80% (v/v) propanol. Chromatograms were coloured with 0·1% ninhydrin solution in chloroform containing 0·1% collidine (Woiwod, 1949).

When new spots, possibly representing peptide material, were found, large-scale chromatograms were run; strips were cut from the papers and sprayed to locate the spots or streaks; horizontal strips were then cut out of the paper as indicated by the guide strips and the material eluted with water. Samples of eluted materials were run again to test the accuracy of the separation. Other samples were hydrolysed for 18 hr. at 105° in 6N-HCl and the hydrolysates taken to dryness at room temperature under reduced pressure; excess HCl was removed by addition of distilled water followed by evaporation to dryness, the process being carried out three times. Examination of the peptides for identification of amino-acids carrying a free amino group was carried out by the deamination method of Consden, Gordon & Martin (1947) and by preparation of dinitrophenyl derivatives according to Sanger (1945, 1946).

When the incubation mixtures contained cysteine, chromatograms showed a number of spots corresponding to partial oxidation products and the spots showed 'tailing'. Treatment of the solutions with bromine water (Consden & Gordon, 1950) oxidizes cysteine to cysteic acid which gives one clearly defined spot. However, during the course of this work it was found that treatment of cysteine with bromine water in the presence of glutamic acid gave rise to a new spot, running between glutamic acid and cysteic acid in butanol-acetic acid, which behaved as though it were a simple dipeptide of cysteic acid and glutamic acid (Van Halteren, 1951). An alternative treatment of cysteine-containing solutions was to allow them to autoxidize by standing in an open vessel for several days. Solutions so treated were preserved by addition of $CHCl_3$. Chromatograms prepared from such solutions showed that cysteine was largely converted to cysteic acid and no new spots were observed as a result of such treatment.

Staph. aureus cells contain high concentrations of certain amino-acids in the free state within the cells (Gale, 1947; Taylor, 1947) and in some experiments small amounts of lysine, glutamic acid or alanine leaked out of the cells in the course of incubation. When this occurred, the spots concerned were also evident in the various control mixtures. Woiwod & Proom (1950) have pointed out that γ-aminobutyric acid may sometimes leak out of the cells. This type of leakage of soluble cell constituents is most marked with staphylococci when incubation takes place in the absence of glucose.

Synthetic peptides. We are indebted to Sir Charles Harington, F.R.S., for a sample of γ-glutamylcysteine; to Dr G. T. Young for α- and γ-glutamylglycine; to Dr R. Consden for α-glutamylalanine, and to Dr W. T. J. Morgan for samples of di-, tri-, and tetra-glycylglycine.

RESULTS

Preliminary work. During investigations of the effect of amino-acid mixtures on the accumulation of free glutamic acid inside *Staph. aureus* (Gale, 1951*b*) it was found that mixtures of three or four amino-acids reduced the rate of accumulation, and that the degree of this reduction was increased by increasing the number of amino-acids in the external mixture. It was decided to test the action of amino-acids, other than glutamic acid, when added one at a time to the experimental mixture of buffered salt, glucose and glutamic acid. In the first place the other amino-acids were added in a concentration equal to twice that of the glutamic acid and the rates of accumulation of free glutamic acid within the cells compared, in each case, with the rate when glutamic acid was the only amino-acid added to the external medium. Table 1 shows the results obtained; the amino-acids fall into three groups: (1) those such as aspartic acid, cysteine, alanine and glycine, which cause a marked decrease in the rate of free glutamic acid accumulation; (2) those which have no marked effect, and (3) those such as valine and leucine which appear to accelerate the rate of free glutamic acid accumulation. L-Alanine has a marked inhibitory action whereas D-alanine is without effect. Aspartic acid is inhibitory whereas asparagine has little effect.

Table 1. *Effect of amino-acids on the rate of accumulation of internal glutamic acid in* Staphylococcus aureus

(Washed suspensions of *Staph. aureus* Duncan, grown for 16 hr. at 30° in deficient medium, incubated in buffered salt solution containing 1% glucose and 1·35 μmol. sodium glutamate/ml. Effect on rate of accumulation of internal free glutamic acid of addition of other amino-acids at concentration of 2·7 μmol./ml. Results expressed as percentage rate in absence of added amino-acids; figures in brackets give range where number of tests was greater than 5.)

Amino-acid added	Rate as percentage control
(1)	
L-Aspartic acid	30 (25–60)
L-Cysteine	40 (10–54)
L-Cystine (sat. soln.)	54 (30–75)
L-Alanine	55 (30–80)
Glycine	65 (30–80)
L-Serine	75
L-Asparagine	82
(2)	
L-Ornithine	97
L-Proline	98
D-Alanine	100
L-Phenylalanine	103
L-Methionine	103
L-Lysine	104
L-Tyrosine	107
L-Tryptophan	107
L-Arginine	107
L-Threonine	110
L-Histidine	110
(3)	
L-Isoleucine	112 (105–118)
L-Valine	117 (106–145)
L-Leucine	118 (105–133)

In the course of more detailed investigations it became clear that the action of aspartic acid was different from that of the other amino-acids in group 1 and, accordingly, this amino-acid will be dealt with separately.

Inhibition of free glutamic acid accumulation by aspartic acid

Effect of concentration. The addition of aspartic acid to the buffered salt solution containing glucose and glutamic acid results in a decrease in the rate of accumulation of free glutamic acid within the cells; if the concentration of aspartic acid is twice that of glutamic acid, then this rate is approximately halved. Fig. 1 shows that the inhibition increases as the concentration of aspartic acid, relative to that of

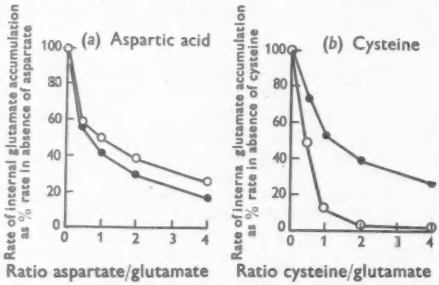
(a) Aspartic acid
(b) Cysteine
Rate of internal glutamate accumulation as % rate in absence of aspartate
Rate of interna glutamate accumulation as % rate in absence of cysteine
Ratio aspartate/glutamate
Ratio cysteine/glutamate

Fig. 1. (*a*) Effect of relative concentrations of aspartic and glutamic acids on rate of accumulation of free glutamic acid within *Staph. aureus*. Washed suspensions of 'deficient' *Staph. aureus* Duncan, incubated in buffered salt solution containing 1% glucose, sodium glutamate at final concentration 1·34 (●) or 10·7 (○) μmol./ml. and sodium aspartate to give ratios indicated by abscissae. Rate of free glutamic acid accumulation within the cells determined over 60 min. at 30°. (*b*) Effect of relative concentrations of cysteine and glutamic acid on rate of accumulation of free glutamic acid within *Staph. aureus*. Conditions as for Fig. 1*a* with substitution of cysteine for sodium aspartate.

glutamic acid, increases. The percentage inhibition was determined for ratios of aspartic to glutamic acid ranging from 0·5 to 4·0 for glutamic acid concentrations of 1·34 and 10·7 μmol./ml.; Fig. 1 shows that the degree of inhibition of the accumulation of free glutamic acid is determined by the ratio of the two amino-acids and not by the absolute concentration of either.

Course of the accumulation of internal free aspartic and glutamic acids. In the first paper of this series (Gale, 1947) it was shown by a non-specific method that aspartic acid could not enter *Streptococcus faecalis* unless glucose was being metabolized; the passage of aspartic acid into the Gram-positive bacterial cell thus occurred under conditions similar to those required for the entry of glutamic acid. This has now been confirmed in the case of *Staph. aureus* by the use of a method specific for aspartic acid (Krebs, 1950). By this method it is possible to determine both aspartic and glutamic acids on the same sample of cells and Fig. 2 shows the rates of appearance of these acids in the free state within *Staph. aureus* cells incubated in the presence of glucose and glutamic acid (curve *A*); aspartic acid (curve *D*); and glutamic acid and one or two equivalents of aspartic acid (curves *B* and *C*). It can be seen that not only does the presence of aspartic acid inhibit the accumulation of free glutamic acid, but that the presence of glutamic acid also inhibits the

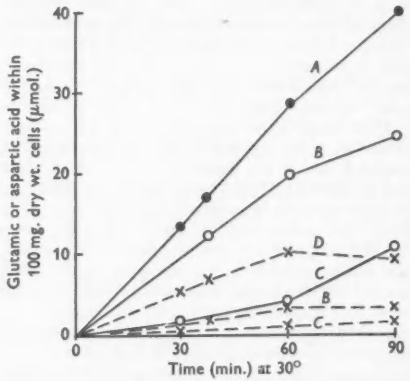

Fig. 2. Accumulation of glutamic and aspartic acids within *Staph. aureus*. Washed suspensions of 'deficient' *Staph. aureus* incubated, at final suspension density = 2·0 mg. dry wt. of cells/ml., in buffered salt solution containing 1% glucose and 1·34 μmol. sodium glutamate/ml. (curve *A*), 1·34 μmol. sodium glutamate + 1·34 μmol. sodium aspartate/ml. (curve *B*), 1·34 μmol. sodium glutamate + 2·7 μmol. sodium aspartate/ml. (curve *C*), 1·34 μmol. sodium aspartate/ml. (curve *D*). Incubation at 30° and samples removed at intervals for estimation of internal concentration of free glutamic acid (continuous lines) and free aspartic acid (broken lines).

accumulation of aspartic acid. When the external concentration of aspartic acid is twice that of the glutamic acid, the sum of the concentrations of the two acids within the cell is markedly less than the concentration of glutamic acid when that amino-acid is the only one present. Consequently each amino-acid must exert some inhibitory action on the accumulation of the other.

Changes in the distribution of free and combined glutamate during incubation with glutamic and aspartic acids. When *Staph. aureus* is incubated with glutamic acid and glucose, free glutamic acid accumulates within the cells and this is drawn from the external medium; the amount of free glutamic acid removed from the medium is invariably greater than the amount which appears within the cells and,

Table 2. *Changes in distribution of free and combined glutamate during incubation of* Staphylococcus aureus *with aspartic acid*

(Washed suspensions of *Staph. aureus*, suspension density = 2·0 mg. dry wt. of cells/ml., incubated for 1 hr. at 37° in buffered salt medium containing 1% glucose, 1·34 μmol. sodium glutamate/ml. with and without 2·7 μmol. sodium aspartate/ml. Results expressed as μmol. glutamic acid/100 mg. dry wt. of cells.)

Incubation mixture ...	Glucose	Glucose + glutamate	Glucose + aspartate	Glucose + glutamate + aspartate	Initial value
Change in internal free glutamate	−4·0	+33·3	+0·2	+10·5	15·6
Change in external free glutamate	+1·7	−53·9	+9·2	−24·7	67·1
Change in combined glutamate of cells	−0·6	− 4·4	−2·9	− 3·6	33·1
Change in combined glutamate of medium	+0·8	+ 0·8	+1·7	+ 1·9	0·0
Balance	−2·1	−24·2	+8·2	−15·9	—

since there is no increase in the combined glutamate of either the cells or the supernatant medium, the glutamic acid which disappears on balance must presumably have undergone metabolism (Gale, 1951*b*; Gale & Mitchell, 1947). In Table 2 the changes in free and combined glutamate of cells and medium which occur when *Staph. aureus* is incubated with glucose and glutamic acid are compared with those that occur when aspartic acid is also present. The effect of the addition of aspartic acid is to reduce, by approximately the same portion, (1) the rate of accumulation of free internal glutamic acid within the cells, (2) the rate of removal of free glutamic acid from the external medium and (3) the rate of disappearance or metabolism of glutamic acid. There is no significant appearance of combined glutamate in the supernatant medium or increase in the combined glutamate of the cells.

When the cells are incubated with glucose and aspartic acid there is no significant increase in the free internal glutamic acid of the cells, but some glutamic acid appears in the external medium; this must presumably arise as the result of transamination (Lichstein & Cohen, 1944).

Chromatographic examination of supernatant media. In all the cases discussed above (Table 2) the supernatant solutions, obtained by centrifuging down the cells at the end of the incubation, were concentrated *in vacuo* and examined by paper chromatography. In no case was it possible to demonstrate the presence of any ninhydrin-positive material other than free glutamic and aspartic acids.

Inhibition by cysteine, cystine, alanine or glycine of accumulation of free glutamic acid

Concentration effects. Fig. 1*b* shows the degree of inhibition of the rate of free glutamic acid accumulation produced by the presence of cysteine at concentrations from one to four times that of the glutamic acid in the external medium. The inhibition increases as the ratio of cysteine to glutamic acid increases. In the case of aspartic acid the inhibition was not affected by the absolute concentration of the amino-acids, but Fig. 1*b* shows that any given ratio of cysteine to glutamic acid has a higher inhibitory action when the concentration of glutamic acid is 10·7 μmol./ml. than when it is 1·34 μmol./ml. Similar effects of absolute concentration were obtained in the cases of inhibition by alanine or glycine.

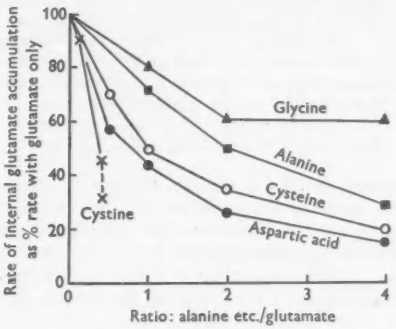

Fig. 3. Effect of amino-acids on rate of accumulation of free glutamic acid in *Staph. aureus*. Washed suspensions of 'deficient' *Staph. aureus* incubated at 30° and final suspension density = 2·0 mg. dry wt./ml. in buffered salt solution containing 1% glucose, 1·34 μmol. sodium glutamate/ml. and one of the above amino-acids at concentrations relative to glutamic acid as indicated by the abscissae. Rate of accumulation of free glutamic acid within the cells determined over 0–60 min. and expressed, in each case, as percentage of the rate when glutamic acid is the only external amino-acid present.

Fig. 3 shows the effect of increasing the concentration of each of the inhibitory amino-acids while keeping the glutamic acid concentration constant. Cystine at low concentrations proves markedly more effective as an inhibitor than cysteine, but solubility considerations prevented its investigation at higher concentrations. The inhibitory effects obtained with aspartic acid and cysteine were reasonably consistent throughout the period covered by these experiments, but the effects of alanine and glycine varied widely from time to time. In the case of

glycine, the concentration necessary to produce 50% inhibition of the rate of accumulation of free glutamic acid varied from three to twelve times that of the glutamic acid.

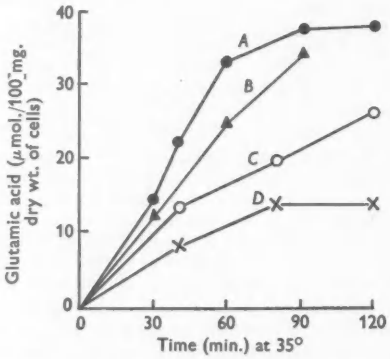

Fig. 4. Effect of glycine, cysteine or cystine on rate of accumulation of free glutamic acid in *Staph. aureus*. Rate of accumulation of free glutamic acid within deficient *Staph. aureus* incubated at 35° in buffered salt solution containing 1% glucose and 1·34 μmol. sodium glutamate/ml. with (*A*) no addition, (*B*) the addition of 2·7 μmol. glycine/ml., (*C*) 2·7 μmol. cysteine/ml., (*D*) cystine to give saturated solution.

Course of the inhibition. Fig. 4 shows the effect of glycine, cysteine and cystine on the rate of the accumulation of internal free glutamic acid. Cysteine and glycine appear to inhibit this rate, but do not decrease the final internal concentration attained. In the case of cystine the inhibition increases with time until, after 2 hr. at 37°, accumulation of free glutamic acid within the cells appears to cease.

Experiments were performed in which the cells were incubated for 1–2 hr. at 37° with glucose and one of the inhibitory amino-acids prior to exposure to glutamic acid and glucose in the absence of other amino-acids. Such pretreatment with cystine for 2 hr. resulted in 30–50% decrease in the subsequent rate of accumulation of free glutamic acid; in no other case was any significant effect of pretreatment observed. It seems possible that the inhibition produced by cystine may be more complex than that due to the other amino-acids investigated.

Changes in distribution of free and combined glutamate. Table 3 shows the changes that occur in the distribution of free and combined glutamate of cells and supernatant medium when *Staph. aureus* is incubated with glucose, glutamic acid and either cysteine or alanine. The presence of either of these amino-acids results in a decrease in the rate of accumulation of free glutamic acid inside the cells, whereas the rate of its removal from the external medium increases. This effect on the external glutamic acid is the opposite of that obtained when aspartic acid is added (Table 2). The amount of glutamate that disappears on balance is slightly increased when cysteine or alanine is present; there is no significant increase in the combined glutamate of the cells, but a significant amount of combined glutamate appears in the external medium. The amount of combined glutamate appearing in the medium is approximately equal to the decrease in the amount of free glutamic acid accumulating within the cells.

Table 3. *Changes in distribution of free and combined glutamate during incubation of* Staphylococcus aureus *with glutamic acid and either cysteine or alanine*

(Washed suspensions of *Staph. aureus*, suspension density = 2·0 mg. dry wt./ml., incubated for 1 hr. at 37° in buffered salt solution containing 1% glucose, 1·34 μmol. sodium glutamate/ml. with 2·7 μmol. cysteine or alanine/ml. as below. Results expressed as μmol. glutamic acid/100 mg. dry wt. of cells.)

Incubation mixture ...	Glucose	Glucose + glutamate	Glucose + cysteine	Glucose + glutamate + cysteine	Initial value
Change in internal free glutamate	−1·0	+28·0	+1·0	+14·7	24·2
Change in external free glutamate	+0·8	−42·2	+0·4	−51·0	67·0
Change in combined glutamate of cells	−2·0	− 3·7	−2·8	− 2·6	35·0
Change in combined glutamate of medium	+0·2	+ 0·8	+1·8	+16·3	0·0
Balance	−2·0	−17·1	−0·4	−23·6	—

Incubation mixture ...	Glucose	Glucose + glutamate	Glucose + alanine	Glucose + glutamate + alanine	Initial value
Change in internal free glutamate	−4·0	+27·1	−1·7	+18·1	30·4
Change in external free glutamate	+3·8	−42·5	+2·7	−58·0	67·1
Change in combined glutamate of cells	−0·6	− 4·2	−4·0	− 4·1	37·2
Change in combined glutamate of medium	+0·8	0·0	+1·6	+15·8	0·0
Balance	0	−19·6	+1·4	−28·2	—

Similar results were obtained when either cystine or glycine was added to the glutamic acid and glucose incubation mixture, although the amounts of combined glutamate formed under these conditions were smaller than when cysteine or alanine was added.

Effect of glucose. The accumulation of internal free glutamic acid does not take place, under the experimental conditions used, unless glucose is present as energy source. The accumulation of combined glutamate occurs, in these experiments, in the external medium and it was therefore of interest to

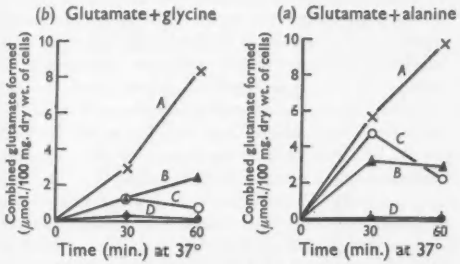

Fig. 5. Effect of glucose on the formation of combined glutamate in the medium during incubation of *Staph. aureus* with glutamic acid and (*a*) alanine or (*b*) glycine. Washed suspensions of 'deficient' *Staph. aureus* Duncan, incubated at 37°, final suspension density = 2·0 mg./ml., in buffered salt solution containing 1·34 μmol. sodium glutamate/ml., 1·34 μmol. alanine (*a*) or 4·0 μmol. glycine/ml. (*b*) and glucose as below. Supernatants collected at 30 and 60 min., evaporated *in vacuo* to one-tenth volume and glutamic acid content determined before and after hydrolysis in 6 N-HCl for 18 hr. Increase in glutamic acid during hydrolysis = combined glutamate and expressed as increase in combined glutamate/100 mg. dry wt. of cells. Initial glucose content of media: (*A*) 0·5, (*B*) 0·2, (*C*) 0·1, (*D*) 0·0 % (w/v).

determine whether glucose was necessary for its production. Experiments were first performed with organisms washed twice in distilled water and then incubated with glutamic acid and one of the inhibitory amino-acids; in no case could the formation of combined glutamate in the external medium or the accumulation of free glutamic acid within the cells be demonstrated. The cells were then incubated with glutamic acid, either alanine or glycine, and amounts of glucose to give initial concentrations of 0, 0·1, 0·2 and 0·5 % (w/v). Fig. 5 shows that a linear formation of combined glutamate occurs throughout 60 min. in the presence of 0·5 % glucose, whereas in the presence of 0·1 % glucose there is an initial formation of combined glutamate which decreases again after the glucose is exhausted in approximately 30 min. It is clear that the presence of glucose is essential for the extracellular formation of combined glutamate in these experiments.

Effect of internal free glutamic acid concentration. When *Staph. aureus* is incubated with glucose and a complete mixture of amino-acids, combined glutamate becomes incorporated in the cellular material and the rate of formation of cellular combined glutamate is dependent on the concentration of free glutamic acid within the cells (Gale, 1951*b*). The question arises whether the internal concentration of free glutamic acid plays any part in the formation of extracellular combined glutamate when the cells are incubated with glucose and either cysteine or alanine. To test this point a batch of cells was prepared in the usual way and divided into two portions; one portion was incubated for 30 min. with glucose and glutamic acid to build up a high internal concentration of free glutamic acid; the other portion was incubated with glucose only. The two suspensions were then washed free from glutamic acid and each was incubated with glucose and either cysteine, glycine or alanine. Table 4 shows that the presence of either

Table 4. *Effect on concentration of internal free glutamic acid of incubation in presence of 'inhibitory' amino-acids*

(Washed suspension of 'deficient' *Staph. aureus* Duncan divided into two portions: each incubated for 30 min. at 37° in buffered salt solution containing (*A*) 1 % glucose, (*B*) 1 % glucose + 1·34 μmol. sodium glutamate/ml. Each then washed twice in distilled water and incubated, at suspension density = 2·0 mg. dry wt. of cells/ml., for 30 min. at 37° in buffered salt solution containing 1 % glucose and other amino-acids as below. Change in concentration of internal free glutamic acid determined during second incubation.)

First incubation in presence of	Second incubation in presence of (μmol./ml.)		Change in internal free glutamic acid (μmol./100 mg. dry wt. of cells)
(*A*) Glucose	Glutamic acid	(1·34)	+35·2
	Glutamic acid + cysteine	(1·34) (2·7)	+28·1
	Glutamic acid + alanine	(1·34) (4·0)	+26·1
	Glutamic acid + glycine	(1·34) (4·0)	+20·6
	(Glucose only)		− 0·9
	Cysteine	(2·7)	− 2·1
	Alanine	(4·0)	− 3·3
	Glycine	(4·0)	− 0·9
(*B*) Glucose + glutamic acid	(Glucose only)		− 9·4
	Cysteine	(2·7)	− 9·4
	Alanine	(4·0)	− 7·3
	Glycine	(4·0)	− 9·1

of these amino-acids in the external medium had little or no significant effect in removing free glutamic acid from within the cells; consequently the free glutamic acid accumulating within the cells cannot readily enter into combination with either cysteine, glycine or alanine outside the cell.

Nature of extracellular peptides formed

Supernatant media obtained in each of the cases discussed above were examined by paper chromatographic methods. The results for each incubation mixture will be discussed separately.

Incubation mixture: glutamic acid + cysteine. Fig. 6 shows the chromatograms given by the supernatant media obtained after incubation of *Staph. aureus* with buffered salt solution, glucose and (II) glutamic acid, (III) cysteine, and (IV) glutamic acid and cysteine. The solutions were treated with bromine water to oxidize cysteine to cysteic acid and the papers run in butanol-acetic acid solvent. In IV a new spot appears, running with an R_F of about 0·10 between glutamic acid and cysteic acid. This new substance does not appear in the supernatant after

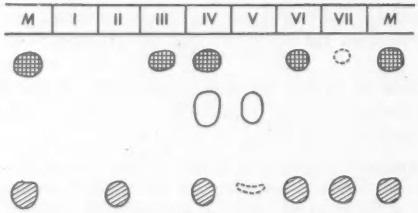

Fig. 6. Diagrammatic representation of paper chromatograms obtained from supernatant media obtained by incubation of *Staph. aureus* with glucose, glutamate and cysteine. Conditions as for Table 3. Concentrated supernatants treated with bromine water and chromatograms run in butanol-acetic acid solvent. *M*, Markers: cysteic acid, ▦; glutamic acid (bottom), ▨; I, supernatant from incubation mixture containing glucose only; II, supernatant from incubation mixture containing glutamic acid and glucose; III, supernatant from incubation mixture containing glucose and cysteine; IV, supernatant from incubation mixture containing glucose, glutamic acid and cysteine; V, material eluted from new intermediate spot in IV; VI, material from V after hydrolysis for 18 hr. at 105° in 6 N-HCl; VII, material from V treated with nitrous fumes before hydrolysis for 18 hr. at 105° in 6 N-HCl.

incubation with glutamic acid only (II) and occasionally appears in trace amounts in the control incubation mixture containing cysteine only (III). (*Staph. aureus* contains high concentrations of free glutamic acid within the cells.) The material corresponding to the new spot has been eluted (V) and hydrolysed, when it gives rise to cysteic and glutamic acids only (VI). The trace of free glutamic acid appearing in V may be due to incomplete separation of the peptide from glutamic acid during the primary separation or to some degree of hydrolysis occurring during the elution and running in acid solvent. If the eluted material is deaminated before hydrolysis, the cysteic acid spot disappears completely or, occasionally, remains as a ghost spot (VII). These results indicate that the new material is a simple peptide of glutamic acid and cysteine, the cysteine carrying a free amino group. This was confirmed by application of Sanger's terminal group method (Sanger, 1945, 1946); treatment of the eluted peptide with fluorodinitrobenzene gave a single spot of the dinitrophenyl derivative with R_F value about 0·6 when run in butanol-acetic acid solvent; on hydrolysis the cysteic acid spot disappeared while the glutamic acid spot remained and gave the usual ninhydrin reaction.

The peptide was run alongside γ-glutamylcysteine and was not identical with the synthetic peptide.

Some doubt was felt concerning the interpretation of these results in view of the artifacts found by Van Halteren (1951) to follow bromine treatment of cysteine in the presence of other amino-acids. However, controlled experiments showed that the amount of material obtained by elution of the new spot in IV was greatly in excess of the amount obtained from a similar position after the 'artifact reaction' only. To confirm the true nature of the biological material, all procedures were repeated using supernatant solutions that had been allowed to autoxidize instead of being treated with bromine water. Separation of the spots was not so clean, but it was possible to repeat and confirm the observations outlined above.

No other new ninhydrin-positive material has been found in the incubation mixture containing glucose, glutamic acid and cysteine.

Incubation mixture: glutamic acid + cystine. Supernatant solutions obtained after incubation of *Staph. aureus* with glucose, glutamic acid and cystine showed, after treatment with bromine water or after autoxidation, a new spot running between cysteic acid and glutamic acid. The new material ran in the same position as that obtained from the incubation mixture containing glutamic acid and cysteine. The yield of material was small, owing to the low solubility of cystine, and no detailed investigations were carried out.

Incubation mixture: glutamic acid + alanine. The chromatograms of supernatants obtained after incubation of *Staph. aureus* with glucose, glutamic acid and alanine showed two new spots running between those of glutamic acid and alanine in either solvent employed (Fig. 7, I: *Y* and *Z*). One of these spots (*Y*) runs with an R_F value slightly less than that of glycine. The material (*Y*) was eluted, run again to ensure that no free glycine was carried with it, and hydrolysed when it split to give glutamic acid, glycine and alanine (Fig. 7, V). None of the components disappeared after deamination or treatment of *Y* with fluorodinitrobenzene before hydrolysis. The second new spot (Fig..7, I: *Z*) runs slightly more slowly than alanine; the material was eluted (Fig. 7, VI) and split on hydrolysis to alanine and

glutamic acid only (Fig. 7, VII). After deamination and hydrolysis, the alanine spot disappeared (Fig. 7, VIII) suggesting that (*Z*) is a simple peptide of glutamic acid and alanine, the alanine carrying the free amino group.

On one occasion a third new spot, running slower than glutamic acid, was obtained (Fig. 7, I: *X*) in propanol-ammonia solvent. On hydrolysis the material obtained from this spot (Fig. 7, II) split to give a well marked glutamic acid spot and a trace of alanine (Fig. 7, III).

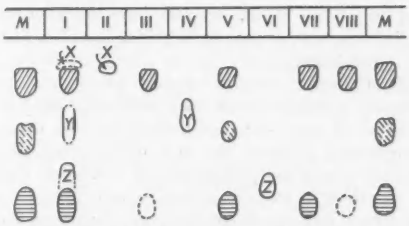

Fig. 7. Diagrammatic representation of paper chromatograms obtained from supernatant media obtained by incubation of *Staph. aureus* with glucose, glutamic acid and alanine. Washed suspensions of 'deficient' *Staph. aureus* Duncan, incubated for 1 hr. at 37° with glucose, sodium glutamate and alanine (conditions as for Table 3). Organisms removed by centrifuging and supernatants concentrated to dryness at room temperature. Chromatograms run in propanol-ammonia solvent. *M*, Markers: glutamic acid, ▨; glycine, ▩; alanine, ▤. I, supernatant from incubation mixture containing glucose, glutamate and alanine; II, material eluted from position I, *X*; III, *X* after hydrolysis for 18 hr. at 105° in 6N-HCl; IV, material eluted from position *Y* in I; V, *Y* after hydrolysis for 18 hr. at 105° in 6N-HCl; VI, material eluted from position I, *Z*; VII, *Z* after hydrolysis for 18 hr. at 105° in 6N-HCl; VIII, *Z* deaminated by treatment with nitrous fumes before hydrolysis and then hydrolysed for 18 hr. at 105° in 6N-HCl.

None of the components *X*, *Y* or *Z* corresponded on the chromatogram with the position of α-glutamylalanine. None of the new spots appeared in control incubation mixtures containing glutamic acid and glucose only, although faint spots were obtained in the positions of *Y* and *Z* when incubation took place with alanine and glucose only.

Incubation mixture: glutamic acid + glycine. Supernatants obtained from incubation mixtures containing glutamic acid, glycine and glucose showed a strong new spot running more slowly than glycine in butanol-acetic acid solvent; in some samples there was evidence of other material running between this spot (Fig. 8, I: *A*) and that of glycine. On elution (Fig. 8, II) and hydrolysis, the material corresponding to the new spot gave rise to glycine only (Fig. 8, III). The spot gave an initial yellow colour when treated with ninhydrin and the colour turned the normal purple on standing. The material of spot *A* was compared, in butanol-acetic acid solvent, with tetra-, tri- and di-glycylglycine. The R_F value of the polyglycine preparations decreased with increasing molecular weight and the material (*A*) had the same R_F value as tetraglycylglycine (Fig. 8, IV–VI). The polyglycine spot appeared whenever *Staph. aureus* was incubated with glucose and glycine. When glutamic acid was also present, the amount of polyglycine decreased when judged by the strength of the ninhydrin reaction on the paper. It has been

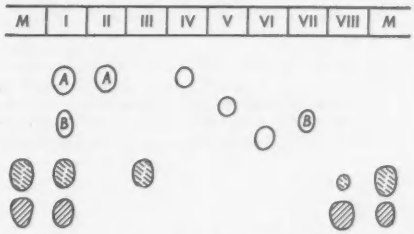

Fig. 8. Diagrammatic representation of paper chromatograms of supernatant media obtained by incubation of *Staph. aureus* with glucose, glutamic acid and glycine. Conditions as for Fig. 7 with glycine substituted for alanine; chromatograms run in butanol-acetic acid solvent. *M*, Markers: glutamic acid, ▨; glycine, ▩. I, supernatant from incubation mixture containing glucose, glutamate and glycine; II, material eluted from position I, *A*; III, *A* after hydrolysis for 18 hr. at 105° in 6N-HCl; IV, tetraglycylglycine; V, triglycylglycine; VI, diglycylglycine; VII, material eluted from position I, *B*; VIII, *B* after hydrolysis for 18 hr. at 105° in 6N-HCl.

mentioned above that the action of glycine on glutamic acid uptake is erratic and the chromatographic results were also inconsistent. A peptide has been isolated from supernatant solutions run in butanol-acetic acid solvent; it runs between the polyglycine and glycine (Fig. 8, I: *B*) and complete separation has not been achieved. On hydrolysis the material eluted from position (*B*) (Fig. 8, VII) liberated glutamic acid and glycine, the increase in the glutamic acid spot being markedly greater than that of the glycine spot (Fig. 8, VIII).

Effects of simple mixtures of amino-acids

Table 5 shows the effect, on the rate of accumulation of free glutamic acid within the cells, of the presence of certain other amino-acids. The inhibition produced by the presence of cysteine is increased by the further addition of cystine, glutathione, alanine or glycine. In general, the inhibitions produced by any of the 'inhibitory group' of amino-acids appear to be additive when these amino-acids are present together. On the other hand, the inhibition produced by either cysteine or alanine is largely released by the further addition of valine or leucine.

This can be correlated with the fact, noted above, that valine and leucine produce an acceleration of the rate of accumulation of glutamic acid within the cell.

Table 5. *Effect of simple mixtures of amino-acids on the accumulation of free glutamic acid within* Staphylococcus aureus

(Washed suspensions of 'deficient' *Staph. aureus* Duncan incubated in buffered salt solution containing 1% glucose and 1·34 μmol. sodium glutamate/ml. with other additions as below. Rate of accumulation of free glutamic acid within the cells determined and expressed as percentage rate occurring in absence of amino-acids other than glutamic acid.)

Amino-acids present in addition to glutamic acid (concentrations in μmol./ml.)	Rate of accumulation of internal free glutamic acid as percentage of that in control without added amino-acids
None	100
Cysteine (1·34)	70
Cystine (sat. soln.)	60
Glycine (4·0)	79
Glutathione (2·7)	91
Cysteine (1·34) + cystine (sat.)	9
Cysteine (1·34) + glycine (4·0)	8
Cysteine (1·34) + glutathione (2·7)	7
Cystine (sat.) + glutathione (2·7)	0
Cystine (sat.) + glycine (4·0)	0
Cysteine (2·7)	54
Alanine (4·0)	72
Valine (2·7)	106
Leucine (2·7)	108
Cysteine (2·7) + valine (2·7)	92
Cysteine (2·7) + leucine (2·7)	94
Alanine (4·0) + valine (2·7)	87
Alanine (4·0) + leucine (2·7)	96

DISCUSSION

When *Staph. aureus* is incubated with glutamic acid in the presence of glucose, the amino-acid enters and accumulates in the free state within the cells. If certain other amino-acids are present, the accumulation of the free glutamic acid within the cells is reduced, but peptides containing glutamic acid accumulate in the external medium. Quantitative analysis of the changes occurring within a given time shows that the amount of combined glutamate accumulating outside the cells as peptide is approximately equal to the reduction in the amount of free glutamic acid accumulating within the cells. Both accumulation of internal free glutamic acid and of external combined glutamate require the presence of glucose. There would appear to be a connexion between the processes involved in the transfer of glutamic acid into the cell on the one hand and in extracellular peptide formation on the other. The fact that the transfer of glutamic acid can be dissociated from glucose fermentation by the action of uncoupling agents (Gale, 1951*a*) has led to the suggestion that some energy-linked metabolism of glutamic acid must take place before or during its transfer across the osmotic barrier of the cell wall. Since the process can be shunted, by the presence of certain other amino-acids outside the cell, to form extracellular peptides, it is possible that peptide-bond formation, or a transpeptidation reaction, may be part of this 'transferring metabolism'. The action of valine and leucine in accelerating the accumulation of glutamic acid, and in releasing the inhibition due to cysteine or alanine, might likewise depend upon the formation of peptides which could penetrate the cell wall more rapidly than the glutamic acid metabolite itself. Experiments are now being carried out with synthetic peptides in order to test this hypothesis.

Identification of the various peptides formed between glutamic acid and cysteine, alanine or glycine has not been completed. In the cases of alanine and glycine, the chromatographic results indicate that a complex system is involved. One of the peptides formed from alanine and glutamic acid appears to be a simple peptide, and the properties of the peptide obtained from glutamic acid and cysteine are consistent with a dipeptide structure; in both cases, deamination and terminal-group tests indicate that the free amino group belongs to the amino-acid other than glutamic acid. Consequently the peptide bond involves the amino group of glutamic acid.

The action of aspartic acid is clearly different from that of cysteine, alanine and glycine. No evidence can be obtained for the accumulation of a peptide of glutamic and aspartic acids. The transfer of aspartic acid into the cell also occurs only in the presence of glucose and it may be that aspartic acid and glutamic acid enter the cell by processes involving a common step so that each appears to compete with the other.

SUMMARY

1. The accumulation of free glutamic acid within *Staphylococcus aureus*, which occurs when the cells are incubated with glutamic acid and glucose, is reduced if aspartic acid, cysteine, alanine or glycine is also present. The presence of either valine or leucine produces an acceleration of the rate of accumulation of free glutamic acid.

2. The inhibition produced by aspartic acid is dependent upon the relative concentrations of aspartic and glutamic acids present. The presence of aspartic acid also reduces the rate at which glutamic acid is withdrawn from the medium. The accumulation of aspartic acid within the cells also requires glucose and is inhibited by the presence of glutamic acid.

3. The inhibition produced by (i) cysteine, (ii) alanine, or (iii) glycine increases as the ratio of each to glutamic acid increases; the inhibition produced

by any given ratio depends also on the absolute concentrations.

4. The decrease in the rate of accumulation of internal free glutamic acid in these cases is accompanied by the formation of combined glutamate in the medium. Chromatographic examination shows that peptide formation has occurred. In the three cases, the extracellular peptides have been hydrolysed and then give glutamic acid and (i) cysteine, (ii) either alanine or alanine and glycine (two peptides), (iii) glycine; a polyglycine is also formed in (iii), but its formation is independent of the presence of glutamic acid.

5. The presence of glucose is essential for the synthesis of these extracellular peptides.

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Studies in Vitamin A

16. PREPARATION OF NEOVITAMIN A ESTERS AND NEORETINENE$_1$

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From the time that the structure of vitamin A was established the possibility had to be admitted that fish-liver oils might contain isomers other than the all-*trans* form, although steric considerations might restrict the number. The most convincing evidence for the existence of *cis-trans* isomers depends: (*a*) on the low yield of crystalline all-*trans*-vitamin A from solutions of the richest concentrates, and (*b*) on the evidence of Robeson & Baxter (1945) that some of the vitamin A in oils and concentrates is slow to react with maleic anhydride. The portion which reacts readily is the all-*trans* form which also crystallizes first. The remainder is neovitamin A, a *cis*-isomer in which only the double bond nearest to the $-CH_2OH$ or $-CH_2OCOR$ group possesses the *cis* configuration. Robeson & Baxter obtained crystalline derivatives of neovitamin A differing from the corresponding derivatives of all-*trans*-vitamin A. They also devised an analytical procedure for determining neovitamin A in fish-liver oils. Their results and those of Meunier & Jouanneteau (1948) indicate that neovitamin A makes up a substantial but variable proportion of the total vitamin A in liver oils. The existence of neovitamin A makes it desirable to know whether the biological activity of fish-liver oils varies with the proportion of the total vitamin A present in the neo form. It is not easy to establish quantitatively a difference between the biological potency of all-*trans*- and neo-vitamin A. Robeson & Baxter failed to find any significant difference, but their experiments may not have been on a scale sufficiently large to establish a small difference.

The existence of neovitamin A also makes it necessary to compare very closely the ultraviolet absorption shown by all-*trans*- and neo-vitamin A. The precise form of the absorption curve is important in the analysis of liver oils, when, as is the rule, irrelevant absorption needs to be allowed for. The simpler procedures for 'correcting' for irrelevant absorption are based on a reference curve for pure all-*trans*-vitamin A and if the vitamin A present in

an oil is spectroscopically heterogeneous, errors may arise depending on the magnitude of differences between the isomers and their relative proportions. In general, *cis-trans* isomerism gives rise to appreciable differences in selective absorption, but such differences cannot be predicted accurately; they need to be determined by experiment. In the case of vitamin A the differences must be slight because there is strong evidence of proportionality between biological activity and intensity of absorption at 328 mμ. for a great range of fish-liver oils; the fiducial limits for biological assays leave room, however, for second order effects which cannot be neglected. It has therefore become important to know how closely the spectrum of neovitamin A in the form of natural esters agrees with that of similar esters of all-*trans*-vitamin A. The original preparation by Robeson & Baxter (1945) depended upon obtaining a very rich concentrate of vitamin A from which the all-*trans* free vitamin A could be crystallized (ethyl formate). The residue after removing the solvent was chromatographed on sodium aluminium silicate. One fraction ($E^{1\%}_{1\,cm.}$, 1410) contained neovitamin A and all-*trans*-vitamin A in the proportions 88 : 12. This material yielded a *p*-phenylazobenzoate which, on recrystallization and hydrolysis, gave crystallizable neovitamin A. The figures in Table 1 enable the two isomeric forms to be compared.

Table 1. *Comparison between all-*trans*-vitamin A and neovitamin A alcohols*

	All-*trans*-vitamin A	Neovitamin A
Melting point ...	62–64°	58–60°
$\lambda_{max.}$ (mμ.) (*iso*propanol)	325	328
$E^{1\%}_{1\,cm.}$ at $\lambda_{max.}$ (*iso*propanol)	1835	1650–1700
Infrared spectra—almost identical		
Biological activity—almost identical		
Anhydro formation—occurs readily, occurs less readily		

According to the maleic anhydride procedure (Robeson & Baxter, 1947; Meunier & Jouanneteau, 1948) various fish-liver oils contained from 18 to 65% of the total vitamin A in the neo ester form. The need to establish the properties of natural neovitamin A esters is thus very real.

METHODS

General

Materials. Some work was done on specially chosen liver oils, but it was generally more convenient to start from ester concentrates made by the Solexol process in South Africa. The materials showed values for $E^{1\%}_{1\,cm.}$ at 328 mμ. of 120–387 and on the whole had extremely little irrelevant absorption.

Adsorbents. Alumina (Peter Spence and Co., Grade 0) was weakened by addition of water (3–10% (w/w) in different experiments). The watered alumina was used the same day.

Solvents. Light petroleum (A.R., b.p. 40–60°) was used in chromatography without purification, but for the more crucial spectrophotometric experiments specially purified material was used. Benzene was used as A.R. quality solvent.

Maleic anhydride (m.p. 50–51°) was purified by sublimation or recrystallized from benzene.

Antimony trichloride reagent. A saturated solution of anhydrous $SbCl_3$ in ethanol-free $CHCl_3$ was used and a little (approx. 1%) acetic anhydride was added just before use.

Spectrophotometry. A Hilger-Nutting visual spectrophotometer was used for the colour test and a Beckman spectrophotometer for the ultraviolet absorption measurements.

Methods of separation

The aim was first to prepare an enriched vitamin concentrate (irrespective of its neovitamin A content) by chromatography on weakened alumina. Little difficulty was experienced in obtaining material of $E^{1\%}_{1\,cm.}$ (328 mμ.) = 600–800. Afterwards, repeated chromatographic separations, depending on the fact that neovitamin A esters are slightly less strongly adsorbed than the all-*trans* esters, permitted gradual enrichment in the neo form. This was at the expense of yield, but it was not necessary from our point of view to obtain large quantities.

Method of analysis for neovitamin A

The procedure of Robeson & Baxter (1947) was slightly modified. A portion of concentrate was accurately weighed and dissolved in benzene. Suitable dilution gave a solution in benzene containing 200–300 i.u./ml. A 5 ml. portion was pipetted into a 10 ml. volumetric flask and made up to volume with a 10% solution of maleic anhydride in benzene. After shaking, the mixture was kept in the dark at 15° for 16 hr. Exactly 1 ml. was then removed and diluted with benzene for use in the $SbCl_3$ colour test. A measured volume of the untreated solution was also tested in the same way. The blue colour ($\lambda_{max.}$ 620 mμ.) was weaker in the solution containing maleic anhydride.

The percentage of the total vitamin A existing as the neo form is given by

$$\frac{R - R_1}{R_2 - R_1} \times 100,$$

where R = percentage recovery of vitamin A in the sample, R_1 = percentage recovery of vitamin A using a pure all-*trans* ester, and R_2 = percentage recovery of neovitamin A ester in a sample of ester free from all-*trans* ester, the criterion of recovery being the appearance of the 620 mμ. maximum in the colour test. The values of R_1 and R_2 are given in Table 2.

Table 2. *Reactivity with* $SbCl_3$ *shown by all-*trans*-vitamin A ester and neovitamin A ester, before and after treatment with maleic anhydride*

(Details of the experimental conditions are given in the text.)

	Relative values		
Material	$E^{1\%}_{1\,cm.}$ at 620 mμ. (no maleic anhydride)	$E^{1\%}_{1\,cm.}$ at 620 mμ. (after maleic anhydride)	Recovery (%)
All-*trans* ester	1·00	0·192	19·2
Neo ester	1·00	0·96	96·0

RESULTS

*Preparation of natural neovitamin A (esters)**

Preliminary chromatographic fractionation. An ester concentrate (1·3 g., *E*, 128) was adsorbed on a column (12 × 1·75 cm.) of weakened alumina (5% water). The chromatogram was developed with light petroleum without suction. The main fraction (0·43 g., *E*, 356) was retained and the residue (0·83 g., *E*, 6) discarded. A second adsorption on a fresh column gave a light petroleum fraction (0·22 g., *E*, 585) and an ethanol eluate (0·165 g., *E*, 3) which was discarded. A third adsorption on a fresh column gave a small anhydrovitamin A fraction which was rejected; the next fraction 3*B* (0·035 g.; *E*, 449) was followed by 3*C* (0·08 g., *E*, 665) and 3*D* (0·04 g., *E*, 757) and 3*E* (0·025 g., *E*, 578). The vitamin of the original concentrate contained 23% neovitamin and 77% all-*trans*-vitamin A. The proportions in 3*B*, 3*C* and 3*D* were 74·5, 26·4 and 7·8% neovitamin A respectively.

The experiment shows the rapid rise in total vitamin A on chromatography. It also shows that neovitamin A is slightly less strongly adsorbed than the all-*trans* form.

Fractionation 1. An enriched concentrate (9·2 g., *E*, 403) containing 15% of its vitamin A as the neo esters was passed through a column (13 × 3 cm.) of weakened alumina (7·5% water). The main fraction (7·7 g., *E*, 527) was passed through a second column (19 × 3 cm.) of weakened alumina (5% water). Two fractions 2*B* (2 g., *E*, 592) and 2*C* (1·75 g., *E*, 761) were retained. These were combined and put through a third column (18 × 1·75 cm., 5% water) and fractions 3*A* (2 g., *E*, 633), 3*B* (0·33 g., *E*, 580) and 3*C* (1·13 g., *E*, 633) were obtained. 3*A* and 3*B* were combined and put through another column yielding 4*A* (0·17 g., *E*, 415) and 4*B* (0·2 g., *E*, 688). Fractions 4*A* and 4*B* contained neovitamin A to the extent of 60 and 40% respectively. The experiment was abandoned at this stage because the degree of enrichment in neovitamin A, though substantial, was not good enough.

Fractionation 2. An ester concentrate (3·3 g., 40% vitamin A esters) gave fractions 1*B* (1·6 g., *E*, 650) and 2*B* (0·73 g., *E*, 781) very rich in total esters. From fraction 3*A* (0·26 g., *E*, 644) fraction 4*B* (0·03 g., *E*, 633) was obtained containing 80% of its vitamin in the neo form. Fraction 3*B* (0·26 g.) gave a first fraction (0·21 g., *E*, 828) which, although made up of about 90% vitamin A esters, only contained 23% in the neo form.

This experiment shows that it is distinctly easier to concentrate the vitamin A esters than to effect a complete separation of the *cis-trans* isomers. The neo percentage was, however, raised to 80 although the yield was small.

Fractionation 3. Two ester concentrates (13 g., *E*, 269; neovitamin 29% and 23·4 g., *E*, 133; neovitamin 24%) were separately chromatographed on weakened alumina (3% water). From them a combined fraction (14·1 g., *E*, 480) was obtained and used as starting point for further work. It gave 1*A* (6·05 g.), 1*B* (1·36 g., *E*, 884) and 1*C* (0·34 g., *E*, 762), 1*B* containing little except mixed vitamin A esters. 1*A*, 1*B* and 1*C* combined gave 2*A* (7·7 g., *E*, 540), which on a fresh column gave 3*A*, 3*B* and 3*C* all with *E*, 540.

* $E^{1\%}_{1\,cm.}$ (325–328 mμ.) is abbreviated below to *E*.

From 3*A* were obtained fractions 4*B* (0·14 g., *E*, 328) and 4*C* (0·5 g., *E*, 602) with 76 and 32% neovitamin A respectively. From 3*A* fractions containing 88 and 42% neo ester were obtained. From 3*C* the best fraction contained 42% neo ester. Combining the 42 and 46% fractions yielded a 70% fraction on further chromatography. The 88 and 70% fractions were combined and put through a further column to yield 6*A* (0·15 g., *E*, 345; 95% neo) and 6*B* (0·105 g., *E*, 460; 72% neo esters).

Fractionation 4. This made use of an enriched concentrate (2·64 g., *E*, 300; 24% neo esters); preliminary fractionations yielded a retained fraction (0·9 g., *E*, 510) from which 1*D* (0·2 g., *E*, 928) was obtained. This on re-adsorption gave a fraction (0·14 g., *E*, 928 with 5% neo ester). Fraction 1*B* in turn gave 2*B* (0·18 g., *E*, 576; 78% neo ester) which in its turn led to 3*B* (0·02 g., 90% neo ester) and 3*C* (0·1 g., *E*, 766; 80% neo ester). It still proved impossible to obtain the neo ester entirely free from the all-*trans* form.

Fractionation 5. This started from 77 g. of ester concentrate (*E*, 279; 23% neo ester). This material was subjected to an elaborate sequence of chromatographic separations. The earliest (least adsorbed) fractions were discarded whenever they showed the anhydrovitamin A bands, however weakly. Many very rich fractions were discarded because the neovitamin A content was less than 35% of the total. By repeatedly combining fractions enriched in neovitamin A esters relatively to all-*trans* esters two fractions were finally retained (0·75 g., *E*, 317; 91% neo ester; 0·51 g., *E*, 382; 86% neo ester). It seems very difficult to accumulate the neo esters without diluting the product with glycerides. The two fractions combined gave in a final fractionation (i), (ii) and (iii). (i) 0·57 g., *E*, 282; 95% neo esters; (ii) 0·34 g., *E*, 416; 90% neo esters; (iii) 0·05 g., *E*, 753; 89% neo esters.

By combining various specimens of small fractions the best neovitamin A ester preparation showed *E*, 810 and < 5% all-*trans* ester.

Absorption curves. The ultraviolet absorption spectra of the best neovitamin A ester fractions have been carefully measured in various solvents. The results are best displayed on a basis of $E_{max.} = 1{\cdot}00$ and compared with the corresponding curves for all-*trans*-vitamin A acetate (Figs. 1–3). (The curves for the all-*trans*-acetate were measured by Cama, Collins & Morton, 1951.)

The natural neo esters showed relatively to the all-*trans* ester higher absorption from 220 to 280 mμ. and from 330 to 390 mμ. There is apparently a *cis*-peak near 250 mμ. In the region 280–330 mμ. the differences between the neo esters and the all-*trans* esters are small, but not negligible (see p. 47).

Neovitamin A alcohol

Preparation. Various enriched fractions were saponified in the usual manner. It was repeatedly noticed that neovitamin A esters are less readily saponified than the all-*trans* esters, but after refluxing (in the presence of quinol) unsaponifiable fractions were obtained which on chromatography gave neovitamin A in the form of an oil *E*, 1600 and $\lambda_{max.}$, 326–327 mμ., in *cyclo*hexane. The

material could not be crystallized. Repeated attempts to utilize the difficult saponification of neovitamin A esters as a means of enrichment were only partially successful, and the formation of artifacts defeated the project.

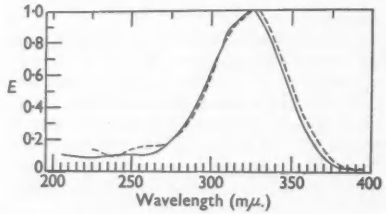

Fig. 1. Absorption curves in light petroleum of: ——, all-*trans*-vitamin A acetate; – – – –, neovitamin A esters (natural). Both curves plotted on a scale where $E_{max.} = 1{\cdot}00$.

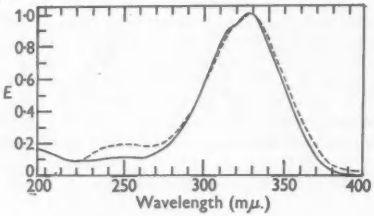

Fig. 2. Absorption curves in *cyclo*hexane of: ——, all-*trans*-vitamin A acetate; – – – –, neovitamin A esters (natural). Both curves plotted on a scale where $E_{max.} = 1{\cdot}00$.

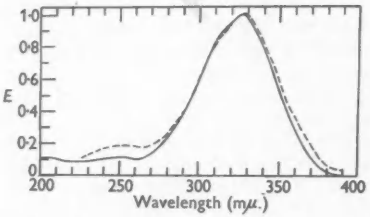

Fig. 3. Absorption curves in ethanol of: ——, all-*trans*-vitamin A acetate; – – – –, neovitamin A esters (natural). Both curves plotted on a scale where $E_{max.} = 1{\cdot}00$.

Absorption curves. Plotted on the basis of $E_{max.}$ 1·00, the curves for neovitamin A alcohol showed higher intensity of absorption from 220 to 290 mμ. and 330 to 390 mμ. as compared with the all-*trans* free vitamin A. The differences, though small, are quite sufficient to influence corrections for irrelevant absorption if neovitamin A predominates over the all-*trans* form.

Irradiation of neovitamin A

The action of light on neovitamin A alcohol or ester resulted at first in a small increase in intensity of absorption, possibly due to formation of some of the all-*trans* isomer.

On exposure to ultraviolet light, ethanolic solutions of vitamin A esters exhibited a sharp increase in green fluorescence. Further irradiation resulted in a decrease, especially if oxygen were not excluded. New inflexions or maxima at 346 and 364 mμ. appeared with a new peak at 275 mμ. and an isosbestic point near 285 mμ. (Sobotka, Kann, Winternitz & Braud, 1944). Similarly, the 328 mμ. maximum of vitamin A acetate in *iso*propanol slowly diminished with the appearance of maxima near 364, 346, 295 and 275 mμ., and if oxygen were excluded the main cause appeared to be light (Bolomey, 1947).

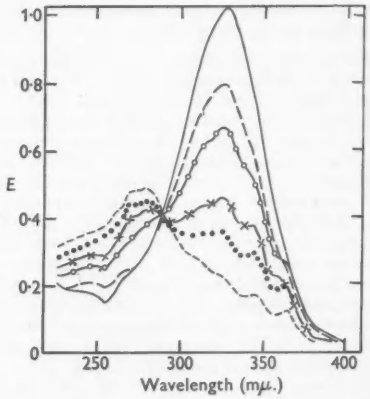

Fig. 4. Absorption curves for irradiated neovitamin A esters ($E^{1\%}_{1\,cm.} = 800$) in ethanol. ——, Zero time; — —, after 3 days in diffuse light; o–o–o, after 9 days in diffuse light; ×–×–×, after 14 days in diffuse light; ....., after 19 days in diffuse light; – – – –, after 28 days in diffuse light.

The effect of light on dilute solutions of vitamin A esters in ethanol has been confirmed in this laboratory using vitamin A concentrates and crystalline vitamin A acetate (unpublished work by Mrs A. W. N. Collins). Exactly similar experiments were carried out using natural neovitamin A esters and the results are shown in Fig. 4.

Oxidation of neovitamin A alcohol to neoretinene$_1$

A neovitamin A ester fraction (0·6 g., E, 342; 95% neo form) was saponified with a trace of added quinol as antioxidant. Chromatography showed that saponification was incomplete and the recovered ester was again treated

with ethanolic KOH under reflux. The total unsaponifiable fraction was oxidized in light petroleum solution by standing over MnO_2 for 12 days in the dark. Purification of the product gave neoretinene$_1$ with $\lambda_{max.}$ 365 mμ. In the colour test $\lambda_{max.}$ occurred at 655 mμ., but moved to 664 mμ. on standing. A preparation on a larger scale showed $\lambda_{max.}$ 365 mμ. (in light petroleum) and 662 mμ. in the colour test.

The differences between retinene$_1$ and neoretinene$_1$ were quite small. As a check, the mother liquors from a first crystallization of a preparation of retinene$_1$ were rechromatographed on alumina, and various portions in repeated operations showed $\lambda_{max.}$ 365 mμ. The neoretinene$_1$ could not be crystallized, but a 2:4-dinitrophenylhydrazone (m.p. 207–208°) and a semicarbazone (m.p. 185°) were obtained.

The absorption maxima for neoretinene$_1$ obtained in various solvents were: light petroleum, 365 mμ.; *cyclo*hexane, 368 mμ.; ethanol, 380 mμ.; chloroform, 385 mμ.; the corresponding figures for retinene$_1$ were 371, 373, 385 and 390 mμ. respectively.

DISCUSSION

The highest intensity of absorption for neovitamin A so far recorded is $E^{1\%}_{1\,cm.}$ (at 328 mμ.) = 1673 (Shantz, 1950). The corresponding figure for all-*trans*-vitamin A alcohol in *iso*propanol is 1835. The difference is not large, but if neovitamin A and all-*trans*-vitamin A are biologically interchangeable, molecule for molecule, the conversion factors for turning spectrophotometric intensities into international units will not be the same. Neither the biological activity nor the molecular extinction coefficient of neovitamin A is as yet sufficiently well established to warrant recommending any complete procedure.

It is evident from Table 3 that so far as the fixation points (Cama *et al.* 1951) are concerned, the displacement is some 2–3 mμ. in the direction of longer wavelengths for neovitamin A as compared with the all-*trans* form. The original fixation points (Morton & Stubbs, 1946) in *cyclo*hexane (313, 328 and 338·5 mμ.) were intermediate between the figures for neo and all-*trans* esters. The differences are least for *cyclo*hexane as solvent and greatest for the free vitamin in *iso*propanol. If the fixation points appropriate to the all-*trans*-vitamin A are used to 'correct' the curve for 100 % neovitamin A esters the result would be about 20 % too low using *iso*propanol and 14 % too low using *cyclo*hexane.

*cyclo*Hexane is thus to be preferred as a solvent in vitamin A determinations. If the light-absorbing solute consists exclusively of the two forms of vitamin A there will be no difficulty in adjusting the correction equations to fit the results of maleic anhydride tests. The problem of highly accurate vitamin A determinations is not, however, a simple one because most oils contain some vitamin A_2 and other products such as kitol, vitamin A epoxide, anhydrovitamin A and other substances contributing irrelevant absorption. It is becoming clear

Table 3. *Fixation points for neovitamin A*

($\lambda_{max.}$ in mμ. and $\lambda\lambda$ at which $E = 6/7\ E_{max.}$. Corresponding figures for all-*trans* shown in black.)

Solvent	λ_1	$\lambda_{max.}$	λ_2
Natural neovitamin A esters			
Light petroleum	312·5	328	337*
All-*trans*	**310**	**325**	**334·9**†
Δ	2·5	3	2·1
*cyclo*Hexane	314·7	329	339·5*
All-*trans*	**312·5**	**327·5**	**337·7**†
Δ	2·2	1·5	1·8
Ethanol	313·7	328	339*
All-*trans*	**311·5**	**326**	**337**†
Δ	2·2	2	2
Neovitamin A acetate			
*iso*Propanol	314·2	328	339·6‡
All-*trans*	**311·7**	**326**	**336·9**†
Δ	2·5	2	2·7
Neovitamin A alcohol			
*iso*Propanol	312·8	328	337‡
All-*trans*	**310**	**324·5**	**334**†
Δ	2·8	3·5	3

* Determinations made in the present investigation.
† Cama *et al.* (1951).
‡ From curves obtained by an American group (privately communicated).

that accuracy will demand a rather elaborate analytical procedure. Extremely careful measurements will first be needed on the purest all-*trans*-vitamin A and on similarly pure neovitamin A. To take advantage of the results it will be necessary to carry out determinations of the all-*trans*-neovitamin A ratio in many liver oils.

For many purposes, however, a simple determination of $E^{1\%}_{1\,cm.}$ max. at 326–328 mμ. (in *cyclo*hexane) would probably be as trustworthy a measurement as any when substances other than vitamin A have been eliminated or allowed for. In the present state of knowledge the conversion factor of 1900 would then be appropriate. The reason for not using correction procedures under such circumstances is that at 326–328 mμ. both forms show rather flat maxima.

So far as our experience goes many natural products contain about 25 % of the total vitamin A in the neo form. The concentrates were surprisingly uniform in this respect, but much more information is needed before a generalization can be made. If, however, this is confirmed it seems likely that for ester concentrates 'over-correction' need not reach 5 %.

The difficulty of saponifying neovitamin A esters

is the only chemical difference we have observed in addition to the difference in speed of reaction with maleic anhydride.

SUMMARY

1. Vitamin A ester concentrates can be enriched by chromatography on weakened alumina until practically free from glycerides, sterol esters and other substances.

2. Repeated chromatographic adsorptions permit the accumulation of neovitamin A esters in the strongly held fractions.

3. Ultraviolet absorption curves for neovitamin A esters closely resemble those of the all-*trans* isomer except that on the long-wave side of 310 mμ. they are displaced by 2–3 mμ.

4. Neovitamin A alcohol yields neoretinene$_1$ on oxidation over manganese dioxide (light petroleum solution). The spectrum of neoretinene$_1$ is slightly displaced in the direction of shorter wavelengths as compared with retinene$_1$.

5. The occurrence of neovitamin A in fish-liver oils may result in over correction in spectroscopic assays if its presence is neglected.

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Studies in Vitamin A

17. SPECTROSCOPIC PROPERTIES OF ALL-*TRANS*-VITAMIN A AND VITAMIN A ACETATE. ANALYSIS OF LIVER OILS

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The adoption by the World Health Organization (W.H.O.) (1950) of pure crystalline vitamin A acetate as the International Standard substance makes important the specification of criteria of purity. As a result of the widespread use of spectrophotometric methods for determining vitamin A in fish-liver oils and concentrates, spectroscopic properties are specially significant. The necessity for 'correcting' the absorption spectra of many natural products so as to allow for 'irrelevant' absorption adds to the importance of accuracy in the data for pure vitamin A. The advent of synthetic vitamin A as an article of commerce (conceivably contaminated by impurities differing from those found in natural products) raises new analytical problems. The existence of neovitamin A (Robeson & Baxter, 1945), a *cis*-isomer of the better known all-*trans*-vitamin A, and its occurrence in fish-liver oils (Meunier & Jouanneteau, 1948) and some synthetic products (Schwarzkopf, Cahnmann, Lewis, Swidinsky & Wuest, 1949) raises issues which have not been fully studied. The simple correction procedure of Morton & Stubbs (1946, 1948) has been very widely used, sometimes with less caution than is required by the plainly stated assumptions on which it rests.

A simple analytical procedure applicable to all kinds of products is no doubt very desirable, but no such method has yet been described. The substances which can contribute 'irrelevant' absorption include: vitamin A_2, kitol, anhydrovitamins A_1 and A_2, oxidation products, carotenoids and unidentified coloured substances, polyene acids and in synthetic products small amounts of intermediates or artifacts. Other disturbing factors include neovitamin(s) A and the effect of variation in the proportion of esterified and free vitamin A.

Although it is not always necessary to aim at high accuracy, the degree of uncertainty in the results obtained by simplified procedures needs to be ascertained.

The present investigation is concerned first with the calibration of the spectrophotometer. This preliminary step is desirable for all accurate work on vitamin A and indispensable in drawing up a specification for the pure material. Secondly, the actual spectra are determined with care in various solvents. Finally, the use to which the spectra are put is illustrated by a full study of a few selected materials, and the whole problem of determining vitamin A is reviewed.

EXPERIMENTAL

Apparatus and materials

Spectroscopic instruments. A new Unicam photoelectric spectrophotometer was used both for determining ultraviolet absorption and for the study of the $SbCl_3$ colour test. A Hilger-Nutting visual spectrophotometer was also used in measuring the blue colour.

Potassium chromate and dichromate. These were used for testing the performance of the spectrophotometer and were of Analar quality.

*Solvents. cyclo*Hexane and ethanol were specially purified for spectroscopy. *iso*Propanol was used as purchased and $CHCl_3$ was left to stand over anhydrous $CaCl_2$ and filtered before use. Light petroleum (b.p. 40–60°) was of A.R. quality.

Vitamin A acetate. Recrystallized material prepared indirectly from fish-liver oils was kindly supplied through Dr J. Matet by Alimentation Equilibrée, Commentry, France. Synthetic vitamin A acetate was kindly supplied through Dr O. Isler by Hoffmann La Roche, Basle, Switzerland.

Vitamin A alcohol. Dr Isler also supplied us with pure crystalline vitamin A alcohol (synthetic).

Vitamin containing oils. The following were used: (*a*) cod-liver oil compound, as supplied to antenatal clinics in England, (*b*) two rich fish-liver oils (species uncertain but probably Spanish tunny liver oils), (*c*) the International Standard preparation of vitamin A acetate issued by the Director of Biological Standards, National Institute for Medical Research, Mill Hill, London, N.W. 7.

Analytical methods

Antimony trichloride colour test. The reagent used was a practically saturated solution (near 15°) of anhydrous $SbCl_3$ in $CHCl_3$ which had been filtered after standing over anhydrous $CaCl_2$ to remove ethanol. The reagent must be filtered, quite clean and colourless, and acetic anhydride (approx. 1 ml./100 ml.) should be added before use. The blue colour produced with vitamin A fades rather quickly, and measurements must be made at a standard time after mixing. In our experiments the ratio of volume of $SbCl_3$ reagent to volume of vitamin A solution (in $CHCl_3$) was from 8:1 to 12:1. The measurements were made on the Unicam spectrophotometer and the total volume of the solution was usually between 3·0 and 3·5 ml. The volume of vitamin A solution was very accurately measured using an 'Agla' Syringe (Burroughs Wellcome and Co.).

It is essential to introduce the $SbCl_3$ reagent quickly from a good quality burette with a wide tip. The cells must be scrupulously clean and the $CHCl_3$ must not be allowed to evaporate from the vitamin A solution while repeated determinations are being made. The spectrophotometer is adjusted (wavelength, dark current, slit-width) after introducing the vitamin A solution into the bottom of the cell; the reagent is then quickly added, the cell holder placed in position and the extinction is read. The reading is made within 8–20 sec. of mixing (average 15 sec.). Under these conditions reproducibility at $\lambda_{max.}$ 620 mμ. is good and the colour is 'caught' very near its peak intensity. To plot the whole curve is laborious since a fresh solution must be taken for measurements at each wavelength every time (Fig. 1). The main difficulties are to prevent a change of concentration by loss of solvent and to keep the cells perfectly clean. To exclude such sources of error small quantities of solution should be poured from a stoppered flask into a stoppered weighing bottle as needed, and the cells should be frequently cleaned with conc. HCl.

Estimation of neovitamin A. Robeson & Baxter (1945) made use of the earlier observations of Kawakami (1935) and Hamano (1937) and others on the formation of maleic anhydride adducts. The method of Robeson & Baxter (1947) has been followed here. It is based upon the much more rapid formation of an adduct with all-*trans*-vitamin A than with neovitamin A.

Maleic anhydride (resublimed) was used as a 10% solution in benzene. A solution in light petroleum of the oil to be tested was prepared and a measured volume containing approx. 3000 i.u. of (total) vitamin A was carefully evaporated and made up to 10 ml. with benzene. A similar solution of crystalline all-*trans*-vitamin A acetate was prepared. 5 ml. portions of each solution were transferred to 10 ml. graduated flasks and made up to the mark with 10% maleic anhydride solution and left in the dark for 16 hr. at 25°. The remaining 5 ml. portions of benzene solution were retained as controls. After 16 hr. the solutions were tested with the Carr-Price reagent. The colour produced with all-*trans*-vitamin A acetate (after maleic anhydride treatment) was feeble and the solution did not require to be diluted, whereas the control solution needed to be suitably diluted. The maleic anhydride treatment reduced the colour intensity to 2% of the control all-*trans* solution (Robeson & Baxter recorded 5%). As no pure crystalline neovitamin A was available, Baxter & Robeson's figure of 90% for the corresponding result for the isomer was accepted. Dalvi & Morton (1951), in this laboratory, obtained essentially similar results at 15° using a neovitamin A ester concentrate. The percentage of neovitamin A in an oil was calculated from the equation

$$\frac{R-R_1}{R_2-R_1}\times 100 = \text{percentage of total vitamin A occurring as the neo isomer.}$$

R is the recovery of vitamin A in the sample expressed as $E^{1\%}_{1\,cm.}$ (620 mμ.) after maleic anhydride treatment, divided by $E^{1\%}_{1\,cm.}$ (620 mμ.) for the control solution and multiplied by 100. [$R_1=2$ and $R_2=90$ (see above).] The degree of reproducibility in the estimates of the neo/all-*trans* ratio is probably a little less than that of the colour test itself. In the case of vitamin A alcohol the factors given by Baxter & Robeson were used, i.e. $R_1=3$ and $R_2=83$.

Estimation of vitamin A and A_2 by the colour test. If the total absorption at 620 mμ. is E_{620} and that at 693 mμ. is E_{693} and if x is the absorption at 620 mμ. due to vitamin A_1 and y is the absorption at 693 mμ. due to vitamin A_2, then

$$E_{620} = x + 0{\cdot}328y,$$

and

$$E_{693} = 0{\cdot}0136x + y.$$

The factor 0·328 is the ratio of E_{620}/E_{693} for pure vitamin A_2 as given by Shantz (1948). Similarly, the factor 0·0136 is the ratio E_{693}/E_{620} for pure vitamin A as shown in Table 7. Hence, solving the equations, we get

$$x = \frac{E_{620} - 0{\cdot}328E_{693}}{0{\cdot}996} \simeq E_{620} - 0{\cdot}328E_{693},$$

$$y = \frac{E_{693} - 0{\cdot}0136E_{620}}{0{\cdot}996} \simeq E_{693} - 0{\cdot}0136E_{620}.$$

Chromatography. The adsorbent was activated alumina (P. Spence and Co., Grade 0) weakened with 8% water. The technique used has been described by Barua & Morton (1949).

Calibration of the spectrophotometer

Potassium chromate and dichromate are perhaps the best known solutes for calibration. The results of our tests compared with data from other laboratories are shown in Tables 1 and 2.

Absorption of vitamin A acetate and alcohol

The melting points of the material were as follows:

'Natural' acetate, 56–57° (uncorr.).
Synthetic acetate, 57–58° (uncorr.).
Mixed sample, 56·5–57·5° (uncorr.).
Synthetic vitamin A alcohol, 57–58° (uncorr.).

The melting points were determined on material from ampoules opened less than 30 min. previously. The recorded higher melting point (62–64°) for vitamin A alcohol is not disputed. We were primarily concerned to make weighings before any deterioration occurred.

Vitamin A acetate has been examined in four solvents. The results are summarized in Tables 3 and 4; Table 5 shows the intensities of absorption as fractions of $E_{max.}$. It is clear that $\lambda_{max.}$ is in the region 325–328 mμ. and $\epsilon_{max.}$ is near to 50,000. The absorption curves all show a very feeble second maximum near 250–253 mμ., $\epsilon_{max.}$ approx. 550.

Synthetic vitamin A alcohol has been examined similarly (Tables 3–5); $\lambda_{max.}$ is from 324 to 327 mμ. and the values of $\epsilon_{max.}$ in the non-polar solvents agree very closely with those for the acetate. The weak secondary absorption peak near 250 mμ. is also shown by the free vitamin. These results are discussed later (p. 56).

Table 1. *Comparison of results obtained by various observers for the maxima and minima in the absorption spectrum of* K_2CrO_4 *in* 0·05 N-KOH

(Light source is hydrogen tube unless otherwise stated.)

Reference	Wavelength (mμ.) (max.)	$E^{1\%}_{1\,cm.}$	Wavelength (mμ.) (min.)	$E^{1\%}_{1\,cm.}$	Wavelength (mμ.) (max.)	$E^{1\%}_{1\,cm.}$	Wavelength (mμ.) (min.)	$E^{1\%}_{1\,cm.}$
a	372	248·5	313	10·3	273	188·5	229	39·1
b	372·5	243·5	313	10·3	273	184·3	229	35
		248†				188·5		39·1
c	372	249	313	10·5	274	191	230	41·8
d	372	248·2	313	10·44	274	192·8	230	37·6
e	371·5	245·2	312·5	10·22	272·5	188·8	227	36·5
		248·8‡						
*f**	371·5	248·2	313·0	10·36	272·5	188·8	229	37·7

* Concn. (%, w/v) 0·0025 0·05 0·0025 0·01

† Hg vapour lamp. ‡ Tungsten filament lamp.

a, Hogness, Zscheile & Sidwell (1937).
b, Zscheile (1947).
c, Gibson (1950).
d, Present authors, Beckman instrument (much used).
e, Riley (1950), new Unicam instrument.
f, Present authors, new Unicam instrument.

Table 2. *Comparison of results on* 0·005% $K_2Cr_2O_7$ *solution (containing a little* H_2SO_4*)*

Wavelength ...	350 mμ. (max.)	313 mμ. (min.)	257 mμ. (max.)	235 mμ. (min.)
Reference	$E^{1\%}_{1\,cm.}$	$E^{1\%}_{1\,cm.}$	$E^{1\%}_{1\,cm.}$	$E^{1\%}_{1\,cm.}$
a	107·6	49·2	144·8	124·8
*b**	107·8	48·6	144·9	124·2$_5$
c	106·7	48·7	145·2	124·8

* Hydrogen source; reversing of cells made no difference except at 257 mμ., where the result given is a mean value. At 313 mμ. the tungsten lamp led to a value of 47·8.

a, Riley (1950).
b, Present authors.
c, Selected data in collaborative tests arranged by the Photoelectric Spectrophotometry Group and analysed by Ridgeman (1950).

Table 3. *Absorption maxima of natural and synthetic all-*trans*-vitamin A*

		Vitamin A acetate				Vitamin A alcohol			
Solvent	Source*	$\lambda_{max.}$ (mμ.)	$\epsilon_{max.}$	f values†	$E^{1\%}_{1\,cm.}$	$\lambda_{max.}$ (mμ.)	$\epsilon_{max.}$	f values	$E^{1\%}_{1\,cm.}$
Light petroleum	*N*	325	52,200	1·42	1590	—	—	—	—
	S	325	52,500		1600	324·5	52,300	1·40	1830
*cyclo*Hexane	*N*	327·5	49,700	1·35	1515	—	—	—	—
	S	328	49,700		1515	326·5	49,800	1·33	1745
*iso*Propanol	*N*	326	50,500	1·34	1540	—	—	—	—
	S	326	50,100		1530	325·5	52,400	1·38	1835
Ethanol	*N*	326	50,300	1·39	1535	—	—	—	—
	S	326	51,000		1560	324	51,500	1·34	1800
Light petroleum	S_I	326	51,000	—	1558	325	51,800	—	1810
*cyclo*Hexane	N_M	328	49,200	—	1500	—	—	—	—
	S_I	328	50,000	—	1525	327	49,300	—	1725
		327·5	49,500	—	1509	326·5	49,500	—	1730
*iso*Propanol	N_M	326	50,500	—	1540	—	—	—	—
	S_I	326	50,200	—	1530	325	52,000	—	1820
		326	49,700	—	1515	324·5	51,800	—	1810
Ethanol	N_M	325·5	50,600	—	1544	—	—	—	—
	S_I	326	50,800	—	1550	325	52,000	—	1820
		325·5	50,100	—	1527	324·5	51,500	—	1800

* N = natural; S = synthetic; S_I = data provided by Dr O. Isler; N_M = data provided by Dr J. Matet.

† f = oscillator strength $= 4{\cdot}31 \times 10^{-9} \int_{25,000\ cm.^{-1}}^{40,000\ cm.^{-1}} \epsilon\, d\nu$.

Table 4. $E^{1\%}_{1\,cm.}$ *values at the absorption maximum of vitamin A acetate and vitamin A alcohol and conversion factors*

Solvent	$E^{1\%}_{1\,cm.}$ (mean values)	Conversion factor*
	Vitamin A acetate	
Light petroleum	1595	1825
*cyclo*Hexane	1515	1920
*iso*Propanol	1535	1895
Ethanol	1550	1880
	Vitamin A alcohol	
Light petroleum	1830	1820
*cyclo*Hexane	1745	1910
*iso*Propanol	1835	1820
Ethanol	1800	1850

* Figures in this column represent defined potency ($2{\cdot}91 \times 10^6$ i.u./g. for acetate, $3{\cdot}33 \times 10^6$ i.u./g. for alcohol) divided by observed $E^{1\%}_{1\,cm.}$ value.

Correction for irrelevant absorption

The correction procedure of Morton & Stubbs (1946, 1948) is based upon the assumption that the irrelevant absorption is linear in the region 310–340 mμ. In its original form (1946) two wavelengths at which E for the pure vitamin A (alcohol or ester) was exactly six-sevenths of $E_{max.}$ were chosen. The present investigation on highly purified vitamin A provided an opportunity to check the three fixation wavelengths in different solvents.

Table 6 shows fixation points and the corresponding correction equations for various solvents. New fixation points (*a*) have been ascertained in which the ratio 6/7 is preserved. The old fixation wavelengths (*b*) (Morton & Stubbs, 1948) for ethanol and *cyclo*hexane are also shown with the necessary formulae permitting correction based on the new absorption curves. For each solvent a third set of fixation points (*c*) with equal wavelength intervals is now added with a suitable and simple correction equation.

Antimony trichloride colour test

It is important to know as precisely as possible the value of $E_{max.}$. The value of $E^{1\%}_{1\,cm.}$ (620 mμ.) for the acetate was 4420; vitamin A alcohol gave a value of $E^{1\%}_{1\,cm.}$ (620 mμ.) of 5070. The corresponding values of the molecular extinction coefficients did not differ significantly and these results were combined. The mean value of $\epsilon_{max.}$ (620 mμ.) was $144{,}900 \pm 870$ (for $P = 0{\cdot}95$; six determinations on the alcohol and nine on the acetate). Table 7 shows the intensity of absorption over the range 520–700 mμ.; there is no inflexion in the region of 580 mμ.

Tests on fish-liver oils

In order to test the applicability of the new spectroscopic data and the proposed new correction formulae, four oils (p. 49) have been examined. In Table 8 are given the experimental results on the first three oils and the results of applying the various correction formulae. The effects of maleic anhydride on the colour test and the calculated amounts of neovitamin A are shown in Table 9, which also shows the absorption at 620 and 693 mμ. and the calculated proportion of vitamins A_1 and A_2 (see Fig. 1).

The high-potency oil B was investigated in greater detail by means of chromatography, and these results are shown in Table 10 and Fig. 2.

International standard preparation

The standard preparation consists of a solution of vitamin A acetate in a selected cotton-seed oil. It is made up in sealed gelatin capsules, each meant to contain 0·250 g. of

Table 5. *Intensities of absorption expressed as fractions of* $E_{max.}$

Vitamin A acetate					Vitamin A alcohol				
Wavelength (mμ.)	*cyclo*-Hexane	*iso*Propanol	Ethanol	Light petroleum	Wavelength (mμ.)	*cyclo*-Hexane	*iso*Propanol	Ethanol	Light petroleum
220	0·091	0·086	0·086	0·086	220	0·086	0·082	0·073	0·081
230	0·096	0·092	0·092	0·090	230	0·090	0·091	0·082	0·089
240	0·106	0·103	0·103	0·100	240	0·102	0·106	0·097	0·099
245	0·111	0·109	0·109	0·103	245	0·108	0·110	0·104	0·105
250	0·114	0·112	0·113	0·104	250	0·112	0·107	0·109	0·111
255	0·115	0·111	0·112	0·103	255	0·111	0·099	0·107	0·112
260	0·109	0·107	0·106	0·104	260	0·107	0·098	0·100	0·106
265	0·112	0·115	0·114	0·117	265	0·108	0·104	0·104	0·112
270	0·132	0·142	0·135	0·152	270	0·130	0·130	0·127	0·137
275	0·165	0·175	0·173	0·189	275	0·161	0·167	0·151	0·177
280	0·215	0·230	0·227	0·247	280	0·212	0·229	0·212	0·236
285	0·270	0·298	0·295	0·320	285	0·274	0·298	0·283	0·312
290	0·350	0·375	0·374	0·401	290	0·346	0·381	0·366	0·396
295	0·448	0·474	0·474	0·507	295	0·448	0·489	0·475	0·505
300	0·555	0·573	0·573	0·602	300	0·553	0·582	0·572	0·605
305	0·670	0·702	0·704	0·731	305	0·669	0·723	0·725	0·740
—	—	—	—	—	309	0·785	0·835	0·830	0·849
310	0·806	0·828	0·828	0·862	310	0·811	0·855	0·845	0·867
311	0·830	0·845	0·849	0·880	311	0·830	0·868	0·860	0·883
312	0·846	0·860	0·866	0·895	312	0·852	0·880	0·873	0·893
313	0·867	0·883	0·878	0·902	313	0·869	0·886	0·884	0·901
314	0·882	0·894	0·897	0·912	314	0·883	0·892	0·895	0·911
315	0·894	0·902	0·902	0·923	315	0·893	0·904	0·900	0·917
316	0·907	0·917	0·915	0·930	316	0·903	0·910	0·909	0·928
317	0·914	0·929	0·924	0·935	317	0·910	0·925	0·922	0·935
318	0·920	0·937	0·938	0·948	318	0·917	0·932	0·932	0·945
319	0·928	0·946	0·948	0·959	319	0·925	0·948	0·947	0·958
320	0·935	0·960	0·963	0·967	320	0·936	0·962	0·958	0·970
321	0·949	0·972	0·973	0·980	321	0·947	0·979	0·975	0·981
322	0·957	0·981	0·985	0·987	322	0·963	0·992	0·985	0·989
323	0·970	0·989	0·994	0·997	323	0·972	0·998	0·990	0·995
324	0·979	0·996	0·998	0·999	324	0·982	1·000	1·000	1·000
325	0·985	0·997	1·000	1·000	325	0·987	1·000	0·997	1·000
326	0·993	1·000	1·000	0·999	326	1·000	0·993	0·995	0·993
327	1·000	0·998	0·998	0·997	327	1·000	0·985	0·983	0·984
328	1·000	0·991	0·994	0·985	328	0·995	0·966	0·971	0·973
329	0·994	0·987	0·989	0·969	329	0·987	0·953	0·956	0·956
330	0·989	0·972	0·973	0·957	330	0·982	0·935	0·936	0·938
331	0·979	0·958	0·959	0·935	331	0·966	0·914	0·917	0·916
332	0·963	0·945	0·946	0·917	332	0·947	0·892	0·898	0·895
333	0·948	0·929	0·924	0·896	333	0·926	0·872	0·879	0·873
334	0·930	0·909	0·904	0·875	334	0·907	0·853	0·857	0·847
335	0·915	0·893	0·896	0·853	335	0·888	0·833	0·837	0·828
336	0·892	0·872	0·871	0·832	336	0·869	0·815	0·822	0·807
337	0·880	0·851	0·855	0·812	337	0·849	0·793	0·802	0·783
338	0·853	0·829	0·829	0·786	338	0·829	0·768	0·774	0·756
339	0·835	0·810	0·815	0·767	339	0·809	0·743	0·760	0·736
340	0·811	0·792	0·795	0·742	340	0·790	0·721	0·736	0·710
345	0·695	0·660	0·667	0·609	345	0·667	0·580	0·591	0·570
350	0·556	0·517	0·520	0·463	350	0·517	0·432	0·442	0·423
355	0·423	0·393	0·393	0·343	355	0·385	0·320	0·330	0·308
360	0·299	0·289	0·292	0·246	360	0·260	0·223	0·233	0·211
365	0·207	0·196	0·197	0·158	365	0·189	0·130	0·140	0·125
370	0·118	0·112	0·113	0·085	370	0·100	0·063	0·070	0·060
375	0·060	0·059	0·058	0·046	375	0·046	0·031	0·035	0·028
380	0·030	0·031	0·034	0·027	380	0·025	0·016	0·019	0·015
385	0·018	0·018	0·018	0·017	385	0·011	0·009	0·010	0·007
390	0·0105	0·011	0·010	0·012	390	0·008	0·005	0·007	0·005
400	0·004	0·003	0·003	0·005	400	0·004	0·001	0·001	0·001

Table 6. *Correction equations for eliminating irrelevant absorption*

(For definition of a and c see text.)

Solvent		E_1	E_2	E_3	$E_{corr.} =$
		(i) Vitamin A acetate (all-*trans*)			
*cyclo*Hexane	(*a*)	312·5	327·5	337·7	$7\ (E_2 - 0{\cdot}405E_1 - 0{\cdot}595E_3)$
	(*b*)*	313	328	338·5	$6{\cdot}58\ (E_2 - 0{\cdot}412E_1 - 0{\cdot}588E_3)$
	(*c*)	316	328	340	$3{\cdot}52\ (2E_2 - E_1 - E_3)$
Ethanol	(*a*)	311·5	326	337	$7\ (E_2 - 0{\cdot}432E_1 - 0{\cdot}568E_3)$
	(*b*)*	311·5	326·5	337·5	$6{\cdot}62\ (E_2 - 0{\cdot}423E_1 - 0{\cdot}577E_3)$
	(*c*)	314	326	338	$3{\cdot}65\ (2E_2 - E_1 - E_3)$
*iso*Propanol	(*a*)	311·7	326	336·9	$7\ (E_2 - 0{\cdot}432_5E_1 - 0{\cdot}567_5E_3)$
	(*c*)	314	326	338	$3{\cdot}61\ (2E_2 - E_1 - E_3)$
Light petroleum	(*a*)	310	325	334·9	$7\ (E_2 - 0{\cdot}399E_1 - 0{\cdot}601E_3)$
	(*c*)	313	325	337	$3{\cdot}5\ (2E_2 - E_1 - E_3)$
		(ii) Vitamin A alcohol (all-*trans*)			
*cyclo*Hexane	(*a*)	312·5	326·5	336·7	$7\ (E_2 - 0{\cdot}422E_1 - 0{\cdot}578E_3)$
	(*b*)*	311	326	336	$6{\cdot}82\ (E_2 - 0{\cdot}4E_1 - 0{\cdot}6E_3)$
	(*c*)	315	327	339	$3{\cdot}36\ (2E_2 - E_1 - E_3)$
Ethanol	(*a*)	311	324·5	334	$7\ (E_2 - 0{\cdot}416E_1 - 0{\cdot}584E_3)$
	(*b*)*	311	325	335·5	$6{\cdot}43\ (E_2 - 0{\cdot}428E_1 - 0{\cdot}572E_3)$
	(*c*)	312	324	336	$3{\cdot}28\ (2E_2 - E_1 - E_3)$
*iso*Propanol	(*a*)	310	324·5	334	$7\ (E_2 - 0{\cdot}396E_1 - 0{\cdot}604E_3)$
	(*c*)	312	324	336	$3{\cdot}28\ (2E_2 - E_1 - E_3)$
Light petroleum	(*a*)	309·5	324	333·6	$7\ (E_2 - 0{\cdot}399E_1 - 0{\cdot}601E_3)$
	(*c*)	312	324	336	$3{\cdot}33\ (2E_2 - E_1 - E_3)$

* Old points (Morton & Stubbs, 1948).

Table 7. *Absorption data relating to the antimony trichloride colour test applied to all-*trans*-vitamin A acetate*

$\lambda_{m\mu.}$	$E^{1\%}_{1\,cm.}$	$\epsilon_{max.}$
520	252	8,300
530	363	12,000
540	532	17,500
550	768	25,400
560	1,120	37,000
570	1,620	53,500
580	2,180	72,000
590	2,900	95,500
600	3,600	119,000
610	4,170	137,000
615	4,350	143,000
620*	4,420±26	144,900±870
625	3,900	129,000
630	3,220	106,000
640	1,740	57,300
650	1,010	33,300
664	325	10,700
670	191	6,300
680	74	2,400
685	47	1,550
693	21	690
700	15	495

Oscillator strength $= 1{\cdot}12 = 4{\cdot}31 \times 10^{-8} \int_{14,500\ cm.^{-1}}^{20,000\ cm.^{-1}} \epsilon\, dv.$

* $E^{1\%}_{1\,cm.}$ 620 mμ. for vitamin A alcohol $= 5070 \pm 30$.

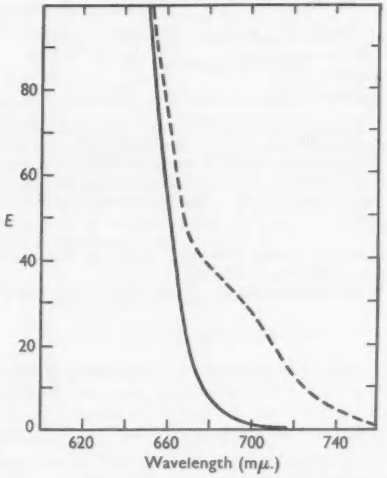

Fig. 1. – – – –, absorption spectrum in the region 650–760 mμ. of the blue solution produced by the interaction of antimony trichloride and high-potency oil B in $CHCl_3$. ——, Vitamin A_1 solution free from A_2. The difference between the two curves is due to the 693 mμ. absorption band shown by vitamin A_2.

Table 8. *Spectroscopic data on fish-liver oils and application of correction procedures*

Material		Solvent	Gross	$E^{1\%}_{1cm.}$ (at $\lambda_{max.}$ of vitamin A) corrected by (a)	(b)	(c)	16 points	M & S*
Cod liver oil compound	(whole oil)	*cyclo*Hexane	0·69	0·537	0·526	0·491	—	0·560
	(unsaponifiable)	*cyclo*Hexane	0·58	0·471	0·458	0·495	—	0·471
High-potency oil A	(whole oil)	*cyclo*Hexane	132	116·4	113·3	112·7	116·2	116·4
	(unsaponifiable)	*cyclo*Hexane	123·6	95·5	97·0	103·2	—	—
High-potency oil B	(whole oil)	*cyclo*Hexane	166·5	142	141	141·5	150	150
	(whole oil)	Light petroleum	177 (169)†	147·7 (141)†	—	150·5 (144)†	—	—
	(whole oil)	Ethanol	169 (166)†	149 (146·5)†	148·5 (146·0)†	149·2 (146·7)†	—	—
	(whole oil)	*iso*Propanol	168·2 (166)†	146·4 (144·5)†	—	142 (140)†	—	—
	(unsaponifiable)	*cyclo*Hexane	160	127	121·5	135·5	141	129

* Correction procedures (*a*), (*b*) and (*c*) explained in text (p. 51). For 16 points correction see Appendix. M & S stands for correction procedure of Morton & Stubbs (1946).
† Equivalent $E^{1\%}_{1cm.}$ in *cyclo*hexane.

Table 9. *Spectroscopic data on fish-liver oils using the colour test showing proportions of neovitamin A and of vitamin A_2*

Material	$E^{1\%}_{1cm.}$ (gross) At 580 mμ.	At 620 mμ.	At 693 mμ.	$E^{1\%}_{1cm.}$ with maleic anhydride 620 mμ.	Percentage of neovitamin A	Calc. $E^{1\%}_{1cm.}$ At* 620 mμ. due to vitamin A_1	At† 693 mμ. due to vitamin A_2
Vitamin A acetate (all-*trans*)	2200	4400	21	67	—	—	—
Cod liver oil compound	0·671	1·31	0·155	0·323	24‡	1·26 (0·432)‖	0·137 (0·049)‖
High-potency oil A	—	357	23	84	25§	349·5 (120)‖	18·1 (6·47)‖
High-potency oil B	263	460	31·7	113	24§	449·5 (154)‖	25·5 (9·12)‖

* Calculated from $E_{620\,m\mu.} - 0{\cdot}328\ E_{693\,m\mu.}$: see text p. 50.
† Calculated from $E_{693\,m\mu.} - 0{\cdot}0136\ E_{620\,m\mu.}$: see text p. 50.
‡ Done on unsaponifiable fraction using the factors for the unesterified vitamin (see text p. 49).
§ Done on whole oil using the factors for the esters (see text p. 49).
‖ Equivalent $E^{1\%}_{1cm.}$ in *cyclo*hexane accepting the following data: vitamin A_1: $\epsilon_{max.}$ (620 mμ.) = 145,000, $\epsilon_{max.}$ (328 mμ.) = 49,700.

Vitamin A_2 (using the data given by Shantz, 1948): $\epsilon_{max.}$ (693 mμ.) = 116,000 and $\epsilon_{max.}$ (351 mμ.) = 41,500 (in ethanol), ϵ (328 mμ.) = 32,750.
It is also assumed that $\epsilon_{max.}$ in *cyclo*hexane is the same as that in ethanol for vitamin A_2.

Table 10. *Spectroscopic properties of fractions obtained by chromatography of high-potency oil B on 8 % weakened alumina*

Eluant	Fraction	$\lambda_{max.}$ (mμ.)	$E^{1\%}_{1cm.}$ (325–328 mμ.) gross	
Light petroleum	1	345, 365, 385	0·12	Anhydrovitamin A
Light petroleum	2	327·5	164*	Vitamin A esters
Light petroleum	3	290	2·3	Kitol or epoxide?
5% Ether-light petroleum	4	None	1·07	—
10% Ether-light petroleum	5	319	4·3	Free vitamin A
Ether	6	None	1·43	—

* Corrected 148·5 equivalent to 144·4 in *cyclo*hexane.

Table 11. *Spectroscopic data on standard vitamin A acetate preparation*

(Solvent: *cyclo*Hexane, 1 cm. cell.)

% (w/v)	Compensator	Extinction expressed as	At 312·5 mμ.	At 313 mμ.	At 316 mμ.	At 327·5 mμ.	At 328 mμ.	At 337·7 mμ.	At 338·5 mμ.	At 340 mμ.
0·1*	*cyclo*Hexane	E (obs.)	0·454†	0·460	0·477	0·522	0·520	0·446†	0·438	0·423
0·1027	*cyclo*Hexane	$E^{1\%}_{1\,cm.}$	4·66†	4·68	4·87	5·29	5·29	4·52†	4·43	4·27
0·1027	0·1027% (w/v) Cotton-seed oil solution	$E^{1\%}_{1\,cm.}$	4·38‡	4·42	4·61	5·09	5·08	4·34‡	4·24	4·09
0·1*	*cyclo*Hexane	$E/E_{max.}$	0·870†	0·881	0·914	1·0	0·997	0·855†	0·839	0·811
0·1027	0·1027% (w/v) Cotton-seed oil solution	$E/E_{max.}$	0·860‡	0·868	0·905	1·0	0·998	0·852‡	0·833	0·803
Expected value for pure vitamin A acetate (see Table 5)		$E/E_{max.}$	0·856‡	0·867	0·907	1·0	1·0	0·858‡	0·844	0·811

% (w/v)	Compensator	Extinction expressed as	At 250 mμ.	At 260 mμ.	At 270 mμ.	At 280 mμ.	At 290 mμ.	At 300 mμ.	At 310 mμ.	At 320 mμ.	At 340 mμ.	At 360 mμ.	At 380 mμ.
0·25*	Solution of cotton-seed oil with *cyclo*hexane	E (obs.)	1·51	0·92	1·03	0·805	0·274	0·124	0·048	0·037	0·020	0·013	0·012

* Nominal concentration: contents of one capsule assumed to be 0·25 g. † Note that these differ. ‡ Note good agreement.

solution, the potency of which should be 10,000 i.u./g. Similar capsules containing the diluent oil only are also issued. The preparation has been treated as if it were a solution of unknown potency. The results are shown in Tables 11 and 12.

Table 12. $E^{1\%}_{1\,cm.}$ *values for the International Standard Preparation in* cyclo*hexane*

	$E^{1\%}_{1\,cm.}$	i.u./g. = ($E^{1\%}_{1\,cm.} \times 1920$)
Expected value	5·21	10,000
Found (compensator *cyclo*hexane) (6 determinations)	5·18±0·078 (P=0·95)	9,950±150
Found (compensator cotton-seed oil in *cyclo*hexane) (5 determinations)	5·03±0·134 (P=0·95)	9,650±260

Fig. 2. Absorption curves of fractions separated from high-potency oil B by chromatography on (8%) weakened alumina. Fraction 1, ×−×−×, intensities are actual × 10²; fraction 2, ——, main ester fraction; fraction 3, •–•–•, 'kitol' (?) intensities are actual × 12·5; fraction 4, ——, intensities are actual × 10; fraction 5, ……, free vitamin A intensities are actual × 8; fraction 6, – – – –, intensities are actual × 10.

DISCUSSION OF RESULTS

Performance of spectrophotometers

Well verified values of extinctions of simple and accessible solutes are extremely scarce, but potassium chromate and dichromate have been much studied and have the advantage of two well separated maxima and minima. If in either curve two out of the four wavelengths are 'correctly' recorded it is probable that the instrument is in good adjustment on the wavelength scale. Solutes with rather broad flat maxima are not, however, ideal for revealing very small errors on the wavelength scale, and a substance with narrow absorption maxima would be

better. In the choice of a calibrating substance, however, it is in general more important to be able to determine intensities of absorption accurately, and in this respect a flatter peak tends to be less influenced by the 'age' of the light source and the consequent variability of slit widths as the output decreases. The two solutes we have used constitute together a severe test of the performance of the instrument.

The results in Tables 1 and 2 show good agreement on the wavelength scale and our own findings on the Unicam instrument appear to be as trustworthy as any. In considering our data on vitamin A, the performance of the spectrophotometer is to be judged by Tables 1 (*f*) and 2 (*b*).

Absorption spectra

Identity of 'natural' and 'synthetic' vitamin A acetate. The absorption curves (in four solvents) for recrystallized vitamin A acetate (all-*trans*) prepared either synthetically or from liver oil concentrates, are indistinguishable. Measurements communicated to us by Dr Isler and Dr Matet show, moreover, that our curves are quantitatively reproducible within very narrow limits of error over the whole spectral range (cf. Chatain & Debodard, 1950).

The behaviour of the two types of product in the antimony trichloride colour test, with and without pretreatment with maleic anhydride, was also identical, showing that both the 'natural' and 'synthetic' acetates were all-*trans* exclusively.

Effect of solvents. The range of $\lambda_{max.}$ (325–328 mμ.) is quite narrow, but the range of molecular extinction coefficients is wider, the highest value being shown for light petroleum, the least polar solvent. We do not recommend its use because it is so volatile that the solution of vitamin A may easily become slightly more concentrated in the course of actual measurements. In *cyclo*hexane $\epsilon_{max.}$ is reproducibly and significantly lower than in *iso*propanol or ethanol (Table 3) (cf. Chilcote, Guerrant & Ellenberger, 1949). It is interesting that the oscillator strength (Table 3), which is a measure of the complete absorption curve from 250–400 mμ., is, within the limits of error, the same for the acetate and the alcohol.

Vitamin A alcohol. Synthetic all-*trans*-vitamin A alcohol shows $\lambda_{max.}$ 324–327 mμ., and in non-polar solvents $\epsilon_{max.}$ agrees extremely well with the corresponding figure for the acetate. In *iso*propanol and ethanol the free alcohol absorbs appreciably more than the acetate (Table 3). This is reproducible and in consequence *cyclo*hexane is a better solvent for use with mixtures of free and esterified vitamin A.

Correction procedures. The samples of vitamin A and vitamin A acetate are undoubtedly a little purer than those formerly available, and the absorption curves are somewhat narrower than those recorded by Morton & Stubbs (1948). The differences though very small, affect corrections for irrelevant absorption and it was necessary to redetermine the fixation points (Table 6). A new correction procedure making use of sixteen wavelengths (see Appendix) greatly reduces the error inherent in the use of three points only, at the cost of much labour. In principle all the correction equations apply to solutions of all-*trans*-vitamin A with superimposed linear irrelevant absorption. The difficulties will be discussed later.

Neovitamin A and the colour test

Once it becomes clear that the absorption spectrum of vitamin A in any given solvent is not entirely fixed and characteristic but will vary (even though only slightly) with the relative proportions of all-*trans*- and neo-vitamin A, the whole analytical problem is moved to a new level of complexity. The antimony trichloride colour test provides through maleic anhydride a means of determining the all-*trans*-/neo-vitamin A ratio. The literature shows very wide variations in the intensity of absorption in the direct colour test, depending partly on the instrument used and partly on the transient nature of the colour. We have therefore taken special care over the measurements recorded in Table 7.

It will be necessary to determine the spectroscopic properties of pure crystalline neovitamin A acetate as carefully as those of the all-*trans* modification, so as to arrive at correction formulae (with appropriate fixation points) applicable to known mixtures of all-*trans* and neovitamin A esters in natural oils. The work of Dalvi & Morton (1951) is a step in that direction.

Vitamin A_2

Vitamin A_2 is present to a greater or lesser extent in most fish-liver oils. This results in some distortion of the spectral absorption curves. The first step in studying vitamin A_2 is to determine $E^{1\%}_{1\,cm.}$ (693 mμ.) in the colour test so that suitable corrections can be made (see p. 50).

Tests on natural products

Test on cod liver oil compound. The gross $E^{1\%}_{1\,cm.}$ (328 mμ.) of 0·69 is too high for the vitamin A present. No anhydrovitamin A is detectable in the absorption, but the colour test shows the presence of vitamin A_2. Tetraene acids which may contribute to the irrelevant absorption are eliminated by saponification, so that the observed $E^{1\%}_{1\,cm.}$ (326 mμ.) of 0·580 on the unsaponifiable fraction is nearer to the true value for the vitamin A_1 contribution. It still needs correction for the vitamin A_2 contribution at 326 mμ. The quantitative colour test shows that $E^{1\%}_{1\,cm.}$ (693 mμ.) (corrected for the overlapping of A_1

and A_2 absorption) is 0·137 which in the direct ultraviolet absorption corresponds with 0·038 at 326 mμ., so that the vitamin A_1 contribution (on the unsaponifiable fraction) is 0·580–0·038 = 0·542. The remaining possible contributors to irrelevant absorption are oxidation products or kitol.

Using the three-point correction procedure on the oil, the average corrected figure is $E^{1\%}_{1\,cm.}$ (328 mμ.) 0·52, and this should allow for all irrelevant absorption provided it is linear over the fixation range. The calculation assumes that the vitamin A_1 is entirely all-*trans*, but experiment shows that 24 % of it occurs in the neo form. In *cyclo*hexane the presence of this proportion of neovitamin A will slightly displace the absorption curve, and it is necessary to multiply 0·520 (see below) by a factor of 1·04 to give 0·542. The fully corrected $E^{1\%}_{1\,cm.}$ (326 mμ.) value is thus < 0·542 but > 0·520.

In the unsaponifiable fraction it is clear that the irrelevant absorption is not strictly linear and application of the three-point correction procedure leads to erroneously low values.

High-potency oil A. The decrease from $E^{1\%}_{1\,cm.}$ 132 to 123·6 represents the 'saponifiable' contribution to irrelevant absorption. The colour test shows the presence of vitamins A_1 and A_2 (Table 9) and the A_1 effect ($E^{1\%}_{1\,cm.}$ (620 mμ.) = 349·5) corresponds with $E^{1\%}_{1\,cm.}$ 120 at 328 mμ. in the ultraviolet test. The A_2 effect in the colour test ($E^{1\%}_{1\,cm.}$ (693 mμ.) = 18·1) corresponds with $E^{1\%}_{1\,cm.} = 5{\cdot}1$ at 328 mμ. Hence the difference 123·6 − 5·1 = 118·5 represents the A_1 contribution at 326 mμ. on the unsaponifiable fraction and agrees well with the value 120 deduced from the colour test. Application of the three-point correction procedure to the oil in *cyclo*hexane leads to a mean value of 114·6. The A_2 contribution will have been eliminated, but the effect of the presence of 25 % of the total vitamin in the neo form will make this figure too low. Experience has shown (Dalvi & Morton, 1951) that the result needs to be multiplied by a factor 1·04, giving 119.

Since the three-point correction procedure applied to the unsaponifiable fraction gives results which are manifestly too low, the combination of variables has made untenable the linearity assumption for irrelevant absorption.

High-potency oil B. The decrease from $E^{1\%}_{1\,cm.} = 167$ to 160 is due to the elimination of saponifiable materials. The colour test shows that the A_1 contribution of $E^{1\%}_{1\,cm.}$ (620 mμ.) = 449·5 (Table 9) corresponds with $E^{1\%}_{1\,cm.} = 154$ for the A_1 contribution at 328 mμ. The colour test for A_2

$$(E^{1\%}_{1\,cm.}\ (693\ m\mu.) = 25{\cdot}5)$$

corresponds with A_2 absorption at 328 mμ. of the value $E^{1\%}_{1\,cm.} = 7{\cdot}2$ (see Fig. 1). Hence the unsaponifiable figure $E^{1\%}_{1\,cm.} = 160$ must be reduced to 152·8, when the A_2 contribution is eliminated. The colour test and the ultraviolet absorption are not, therefore, discordant.

Application of the correction procedures to the whole oil in various solvents leads to a figure of 143·4 (reduced to *cyclo*hexane). This eliminates all irrelevant absorption, but the presence of neovitamin A (24 %) makes it necessary to multiply by 1·04, leading to 149. Chromatography on the whole oil leads to 144·4 (*cyclo*hexane) after correction eliminating vitamin A_2, and this figure will need to be corrected for neovitamin A, leading to 150. The chromatographic separation disclosed the presence of a small free vitamin A fraction which must be added to 150. The chromatographic separation leads to a vitamin A_1 contribution of slightly over 150. The unsaponifiable figure of 152·8 has clearly not allowed for the presence of kitol and must be reduced to not more than 150.

It seems clear that each approach leads to a figure of about 149–150.

Summarizing, we have:

Method	Calculated $E^{1\%}_{1\,cm.}$ (328 mμ.)	Remarks
Colour test	154	Too high, no correction made for oxidation products, kitol, etc.
Chromatograph	150	May be too high if separation incomplete, but needs addition for free vitamin A
Unsaponifiable fraction corrected for A_2	152·8	Not corrected for oxidation products or kitol
Geometric corrections on whole oil corrected for neovitamin A	149	—

Finally, application of geometrical correction procedures to the unsaponifiable fraction leads to erroneously low results and the basis of the correction must be invalid because of neovitamin A and the appearance of irrelevant absorption mainly on the short-wave side of the peak.

International standard preparation of vitamin A acetate

Examination of the standard preparation should be a relatively simple matter because the complications due to vitamin A_2, neovitamin A and oxidation products do not arise. If the properties of pure vitamin A acetate given in Tables 3 and 4 are correct and the standard preparation contains pure all-*trans*-acetate and is stable, then it should be possible to find 10,000 i.u./g.

When the solvent is *cyclo*hexane the expected intensity of absorption will be 10,000/1920 (Table 4) = $E^{1\%}_{1\,cm.}$ 5·21. The observed value using *cyclo*hexane

only in the compensating cell is not significantly different from the expected value. The sounder method of compensation with the diluent oil gives a result which is significantly lower (Table 12). The assumption that each capsule contains exactly 0·250 g. of material may not necessarily be correct, but even when the oily solution was carefully weighed out, $E^{1\%}_{1\,cm.}$ (328 mμ.) was low (5·09) with compensation using the same concentration of diluent oil. It is clear that the vitamin A acetate has not been appreciably oxidized because the fixation points are correctly recorded.

An interesting point emerges if the standard preparation is first saponified and the unsaponifiable fraction dissolved in *iso*propanol. It will be noted that in *iso*propanol $\epsilon_{max.}$ for vitamin A acetate is near 50,300 and that of vitamin A alcohol is 52,400 (Table 3). The expected $E^{1\%}_{1\,cm.}$ value for the international standard preparation would be 5·28 and that of an equivalent solution of vitamin A alcohol 5·49. To neglect the rise in E value would imply accepting as containing 10,000 i.u./g. a solution which actually contained 9620 i.u./g. This method of testing would fit the recommendation of the U.S. Pharmacopoeia (1950) and errors might pass undetected.

It seems very probable that the samples of vitamin A acetate used in the present work are a little purer than earlier ones including that used for the standard preparation.

CONCLUSIONS

The method of determining vitamin A in fish-liver oils and other products from the intensity of ultraviolet absorption is well tried and serviceable. The major obstacle in the way of accuracy is irrelevant absorption, but recent work shows that the co-existence of all-*trans*- and neo-vitamin A also gives rise to difficulty.

The work of Morton & Stubbs (1946, 1948) showed how, in favourable circumstances, the total irrelevant absorption could be allowed for. Some of the substances contributing to the irrelevant absorption, e.g. kitol and vitamin A epoxide can be removed by chromatography. In many cases (e.g. cod liver oils) preparation of unsaponifiable extracts removes much of the irrelevant absorption, but with high-potency materials the effect is much less noticeable and subsequent chromatography fails to remove vitamin A_2 and some other 'impurities'. The more elaborate the chemical manipulation the greater will be the risk of loss.

The spectra of all-*trans*- and neo-vitamin A are sufficiently different to cause appreciable distortion of the curve of either when mixtures are studied. The maleic anhydride procedure applied to the colour test permits determination of the relative proportion of all all-*trans* and neo isomers, and whenever the Morton-Stubbs or similar procedures suggest the need for a large correction for irrelevant absorption (particularly in high-potency oils) the proportion of neovitamin A should be determined.

It also seems necessary to look for vitamin A_2 as a routine. This means examining the solution by the antimony trichloride colour test for a band or an inflexion near 693 mμ. Indeed much is gained by determining $E^{1\%}_{1\,cm.}$ at both 620 and 693 mμ. quantitatively and correcting for overlapping absorption (see p. 50). The ultraviolet absorption at 328 mμ., due to vitamin A_2, must then be calculated and subtracted from the gross $E^{1\%}_{1\,cm.}$ (326–328 mμ.) on the oil or the unsaponifiable fraction.

Application of the correction equations (Table 6) will be valid when the vitamin is entirely all-*trans*, and as soon as data for crystalline neovitamin A acetate comparable with those in Table 5 for the all-*trans* form are available, new equations to fit any ascertained mixture of the isomers can be deduced. An alternative is to correct for irrelevant absorption using the equations based on the all-*trans* form and afterwards to adjust for the presence of known proportions of neovitamin A as has been done in the present paper.

It is also theoretically possible, using four points, to construct an equation that would enable the amounts of all-*trans*- and of neo-vitamin A to be determined, provided all the remaining absorption in the region 310–340 mμ. is linear. The errors would, however, be large and if sixteen wavelengths were used, and a solvent in which the absorption of all-*trans*- and neo-vitamin A differed most (*iso*propanol?), then the errors should be no greater than those arising in the other methods available. The equation would be a simple extension of that given in the Appendix, but would involve a knowledge of the absorption spectrum of pure neovitamin A acetate. In a trial the use of four points led to 29% neovitamin A when the maleic anhydride procedure indicated 24%.

It is evident that the correction equations (Table 6) can be applied much more successfully to whole oils (in *cyclo*hexane) than to unsaponifiable fractions. *cyclo*Hexane is a more suitable solvent than *iso*propanol because the difference between the spectra of the two isomers is least in the hydrocarbon solutions.

The three oils which have been studied in detail can be corrected for irrelevant absorption by different combinations of methods, all of which approach the same value in each case. The special difficulty with the unsaponifiable fractions arises from the fact that the main irrelevant absorption is not linear in the region 310–340 mμ.; it seems in fact to be considerable at 310 mμ. but not from 326 to 340 mμ. We have reason to suspect isomerization during saponification.

The outcome of this investigation is: (*a*) For low-potency oils of good quality the accepted use of un-

saponifiable extracts will give a 'normal' absorption curve the $E^{1\%}_{1\,cm.}$ value of which needs to be corrected only for vitamin A_2; the 'corrected' figure on the oil will agree closely if adjusted for the presence of neovitamin A. (*b*) For higher potency oils, application of two or more of the correction formulae will give a result which, when adjusted for the presence of neovitamin A, will be practically the same as that resulting from the most laborious elimination one by one of interfering substances.

The analyst must at present treat vitamin A_2 as a contaminant, but a time may come when it will be legitimate to include its activity, apparently 40 % of that of an equal weight of vitamin A_1 (Shantz & Brinkman, 1950). That would depend on the unknown capacity of man to utilize vitamin A_2 and presumably on official recognition.

SUMMARY

1. The photoelectric spectrophotometer was calibrated with potassium chromate and dichromate solutions. The results provide a basis for testing the performance of other instruments.

2. Natural and synthetic vitamin A are indistinguishable. The values of $E^{1\%}_{1\,cm.}$ and $\lambda_{max.}$ for vitamin A acetate and for vitamin A alcohol were obtained in *cyclo*hexane, light petroleum, ethanol and *iso*propanol. The values of $\epsilon_{max.}$ in *cyclo*hexane and light petroleum were the same for the free acetate and for the alcohol. The values of $\epsilon_{max.}$ in ethanol and *iso*propanol for the alcohol were, however, higher than for the ester.

3. All the absorption curves showed a second maximum at about 250–253 mμ., $\epsilon_{max.}$ approx. 550.

4. The value of the factor for converting $E^{1\%}_{1\,cm.}$ to i.u./g. is not strictly constant, but varies with the solvent and the state of combination of the vitamin A.

5. The complete absorption curve for the reaction product of vitamin A and antimony trichloride has been obtained. The value of $\epsilon_{max.}$ is $144{,}900 \pm 870$ at 620 mμ.

6. A cod liver oil preparation, two high-potency oils and the International Standard Preparation of vitamin A acetate have been studied in detail. The latter is perhaps not quite up to specification.

7. Although for a wide range of products the $E^{1\%}_{1\,cm.}$ value at 325–328 mμ. on the oil or the 'unsaponifiable fraction' is a good guide to potency, it is necessary in aiming at an accurate assay to take full account of the presence of anhydrovitamins A, vitamin A_2, kitol, epoxides and other substances. Except with low-potency oils it is almost always safer to work on the whole oil because the correction procedure when applied to unsaponifiable materials tends to give low results. It is also necessary to determine the neo/all-*trans* ratio. This means that in addition to the main test based on ultraviolet absorption, a number of subsidiary tests are essential. The procedure is discussed fully and the results are compared with 'corrected' values using the equations described.

We are indebted to the Medical Research Council for a grant to the Department. One of us (F.D.C.) is the holder of an I.C.I. Fellowship. The gift of numerous samples of highly purified material from MM. Alimentation Equilibrée, France, and Hoffmann La Roche, Switzerland, is gratefully acknowledged.

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APPENDIX

Correction Procedure

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A correction procedure can be developed which makes use of all the wavelengths at 2 mμ. intervals from 310 to 340 mμ. As only three wavelengths are necessary in order to correct for linear irrelevant absorption, thirteen degrees of freedom are available for the determination of the error. The following formulae are derived by the method of least squares (see Whittaker & Robinson, 1924).

Let E be the measured extinction at wavelength λ and let α be the ratio of the absorption of pure all-*trans*-vitamin A λ to the absorption at wavelength $\lambda_{max.}$ Then at wave-at length λ

$$E = \alpha\bar{E} + a\lambda + b,$$

where $\bar{E}$ = extinction actually due to vitamin A at $\lambda_{max.}$ and a and b are constants.

By the method of least squares

$$\bar{E} = \frac{\begin{vmatrix} \Sigma\alpha E & \Sigma\alpha\lambda & \Sigma\alpha \\ \Sigma\lambda E & \Sigma\lambda^2 & \Sigma\lambda \\ \Sigma E & \Sigma\lambda & 16 \end{vmatrix}}{\begin{vmatrix} \Sigma\alpha^2 & \Sigma\alpha\lambda & \Sigma\alpha \\ \Sigma\alpha\lambda & \Sigma\lambda^2 & \Sigma\lambda \\ \Sigma\alpha & \Sigma\lambda & 16 \end{vmatrix}}.$$

The variance of E will be

$$\sigma^2_{\bar{E}} = \frac{\begin{vmatrix} \Sigma\lambda^2 & \Sigma\lambda \\ \Sigma\lambda & 16 \end{vmatrix} \begin{vmatrix} \Sigma\alpha^2 & \Sigma\alpha\lambda & \Sigma\alpha & \Sigma\alpha E \\ \Sigma\alpha\lambda & \Sigma\lambda^2 & \Sigma\lambda & \Sigma\lambda E \\ \Sigma\alpha & \Sigma\lambda & 16 & \Sigma E \\ \Sigma\alpha E & \Sigma\lambda E & \Sigma E & \Sigma E^2 \end{vmatrix}}{13 \times \begin{vmatrix} \Sigma\alpha^2 & \Sigma\alpha\lambda & \Sigma\alpha \\ \Sigma\alpha\lambda & \Sigma\lambda^2 & \Sigma\lambda \\ \Sigma\alpha & \Sigma\lambda & 16 \end{vmatrix}^2}.$$

In the case of vitamin A acetate in *cyclo*hexane,

$$\bar{E} = \frac{21760\Sigma\alpha E - 19{\cdot}824\Sigma\lambda E - 13488\Sigma E}{1297{\cdot}422}.$$

In the case of vitamin A alcohol in *cyclo*hexane,

$$\bar{E} = \frac{21760\Sigma\alpha E + 2{\cdot}848\Sigma\lambda E - 20735{\cdot}36\Sigma E}{1461{\cdot}4985}.$$

The above derivation can be generalized and would include the Morton-Stubbs equations.

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Separation and Estimation of Saturated C_2–C_7 Fatty Acids by Paper Partition Chromatography

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Investigations at this laboratory, involving many analyses of mixtures of lower fatty acids in rumen contents and in blood of sheep, indicated the need for more convenient methods than those at present available. Preliminary investigations have indicated that paper chromatography offers a solution to this problem.

One of us (R.L.R.) had previously used the silica-gel partition-chromatographic methods of Elsden (1946) and of Moyle, Baldwin & Scarisbrick (1948). Elsden's method satisfied a long-felt need, but it has certain disadvantages. Moyle *et al.* (1948) found that the use of buffered columns obviated most of the disadvantages of Elsden's method, and gave extremely accurate results. However, it is tedious, and this is an important limiting factor in routine analyses. Particularly is this so when more than three acids are to be determined, and hence two or more columns must be used.

The method of Peterson & Johnson (1948) also employs silica-gel columns, but is likewise tedious because more than one column must be used to effect separation of the acids and many titrations are necessary.

Paper chromatography has recently been applied to the separation and quantitative determination of relatively non-volatile organic acids (Lugg & Overell, 1947, 1948) and to the separation of aromatic monocarboxylic acids (Lederer, 1949*b*). The volatility of the lower fatty acids, however, limits the direct application of paper-chromatographic techniques for their separation and identification. Fink & Fink (1949) overcame this difficulty by chromatographing the acids as their hydroxamate derivatives and spraying the developed chromatogram with ferric chloride. The derivatives were then visible as purple spots on a yellow background, but accurate quantitative determination was not possible.

We have now devised a paper partition-chromatographic procedure by which we can separate, identify and estimate lower fatty acids from C_2 to C_7 in the one procedure and with sufficient accuracy for most biological purposes. Since our work was completed, Brown & Hall (1950; cf. Brown, 1950) have published a very similar method for the separation and identification of lower fatty acids, but their findings differ from ours in certain aspects.

Lactic and formic acids cannot be separated from acetic by our technique, or by that of Brown & Hall (1950). These two acids, when present in significant amounts, must be removed by preliminary distillation by a method such as that of Friedemann (1938). When determining volatile fatty acids in rumen liquor, in which lactic and formic acids are not usually present in significant quantities, a single Markham steam distillation (McAnally, 1944) is adequate.

The chromatographic technique which we employ is the ascending development for 20–24 hr. as described by Williams & Kirby (1948). The solvent is butanol saturated with aqueous 1·5N-ammonia, which was used previously by one of us in the chromatography of inorganic anions (Lederer, 1949*a*) and also by Brown & Hall (1950). The acids are run on the chromatogram in the form of their ammonium salts. Unlike Brown & Hall (1950), we have found that the use of sodium salts is unsatisfactory because, under most conditions, the spot given by the sodium ion partly or completely obliterates that given by acetate.

After development and air-drying, the paper is sprayed with bromocresol purple in ethanol containing formaldehyde. The inclusion of formaldehyde in this reagent increases the subsequent spot definition. Formaldehyde reacts with the ammonium ion to form the weak base hexamethylenetetramine. The free acids, after exposure of the chromatogram to ammonia, appear as regular yellow spots on a purple background. In our hands, bromocresol purple has proved superior to several other indicators, including bromocresol green and bromothymol blue, which were used by Brown & Hall (1950). It is more sensitive to low concentrations of the acids and gives spots which are more sharply defined and usually more stable; these considerations, as will be seen, are important in quantitative analysis.

Of the methods of quantitative analysis investigated, the only one found suitable was the calculation of acid content from spot area, the latter being related linearly to the logarithm of the former. The use of this technique was suggested by Fisher, Parsons & Morrison (1948) for the quantitative determination of amino-acids and sugars. Bryant & Overell (1951) have successfully used the relationship in the estimation of relatively non-volatile organic acids, and their observations are confirmed in our work with lower fatty acids.

EXPERIMENTAL

Reagents

Solvent system. Redistilled *n*-butanol is saturated with an equal volume of aqueous 1·5N-NH_3.

Indicator solution. This is a solution of 0·04% (w/v) bromocresol purple in a 1:5 (v/v) dilution of B.P. formalin in ethanol. The pH of this solution is adjusted to approximately 5·0 by the addition of 0·1N-NaOH.

General procedure

Development of chromatogram. Sheets of Whatman no. 1 (special chromatographic) paper (12 in. (30 cm.) square) are exposed to an atmosphere of concentrated aqueous NH_3 in a closed vessel for 3–4 hr. immediately before use. Samples of approximately 5 μl., containing the fatty acids in the form of their ammonium salts, are placed 3–4 cm. apart along a 'starting line' 3 cm. from the bottom of the paper. Each sheet is stapled in the form of a cylinder which is then stood in a 0·5–0·7 cm. layer of butanol phase of the solvent in a convenient jar or tank with close-fitting lid. The aqueous solvent phase is transferred to a beaker or beakers which are stood in the butanol phase. The same solvent mixture can be used for at least four chromatograms. A development time of 18 hr. is sufficient for complete separation of the acids, the solvent reaching the top of the paper in 18–20 hr. The time of development is not critical and can be extended to at least 24 hr. if necessary. When development is complete, the paper is removed and allowed to dry at room temperature.

Spraying technique. Several attempts were made to control the spraying intensity in an effort to obtain even spraying of the paper. With light spraying, any unevenness causes erratic quantitative results. We found, however, that the best spot definition was obtained with an intensity of spraying sufficient to allow complete and even penetration of indicator solution to the reverse side of the paper. For this purpose an ordinary atomizer proved as efficient as any other technique tested.

Development of acid spots. After holding the sprayed chromatogram for 2–3 min. in an atmosphere saturated with a 3% (v/v) dilution of aqueous NH_3 (sp.gr. 0·880) (removing it for a few seconds at brief intervals), the acids appear as bright-yellow spots on a stable purple background (Fig. 1).

Quantitative analysis. The acid spots are traced on to graph paper, ruled in 0·125 in. (approx. 3 mm.) squares, and an estimate of their area is thus obtained. Two standard solutions, containing known concentrations of the ammonium salts of the fatty acids under investigation, must be chromatographed on the same sheet with each test solution.

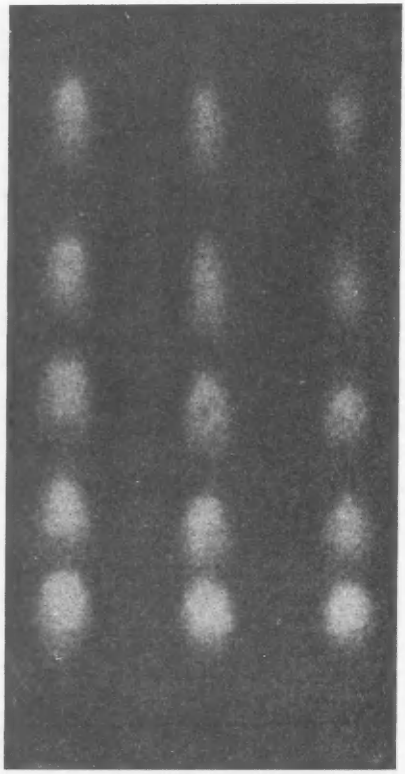

Fig. 1. Separation of C_2–C_6 fatty acids from 5μl. samples of mixed solutions of their ammonium salts. Concentration of acids (μg./5 μl.) from left to right: acetic: 93, 78, 62; propionic: 77, 64, 51; *n*-butyric: 76, 63, 50; *iso*valeric: 68, 57, 45; *n*-hexanoic: 64, 54, 43 respectively. The chromatogram was run for 18 hr. on Whatman no. 1 (special chromatographic) paper, with *n*-butanol-aqueous ammonia as the developing solvent (see Text).

Due to the direct relationship between spot area and logarithm of acid content, the spot areas given by these standards can be used to plot regression lines for each acid, from which the acid content of unknown spots can be read directly.

Normally we run two standards with six or seven different unknowns on each of four sheets. Calculations are thus based on the mean of quadruplicate estimates and variations from sheet to sheet are automatically accounted for. The concentrations of each acid in the standard solutions are so adjusted that test solutions fall within their limits.

RESULTS

R_F values

The R_F values of the ammonium salts of lower fatty acids, from formic to nonanoic (pelargonic), are shown in Table 1. Acids from acetic to heptanoic (oenanthic) (C_2–C_7) separate completely, but heptanoic acid (C_7) is inseparable from octanoic (caprylic; C_8) and from nonanoic acid (C_9). Octanoic acid can be estimated in the presence of hexanoic (caproic) acid (C_6) but not when heptanoic or nonanoic acids are also present. Formic and lactic acids cannot be separated from acetic acid, but preliminary steam distillation (Friedemann, 1938) prevents their interference in acetic acid determinations. *n*-Valeric and *iso*valeric acids are inseparable. The ammonium salts of all dicarboxylic acids investigated did not move in this solvent.

Table 1. *R_F values of ammonium salts of fatty acids and of other organic acids*

(Development with *n*-butanol-aqueous ammonia by upward running technique with Whatman no. 1 filter paper, see Text.)

Acid	R_F values: Single acids in solution	R_F values: Mixture of acids in solution
Formic	0·10	—
Acetic	0·11	0·10–0·11
Propionic	0·19	0·18–0·20
n-Butyric	0·29	0·29–0·32
n-Valeric	0·41	—
*iso*Valeric	0·40	0·39–0·44
n-Hexanoic (caproic)	0·53	0·53–0·59
n-Heptanoic (oenanthic)	0·62	0·62–0·64
n-Octanoic (caprylic)	0·65	0·64–0·66
n-Nonanoic (pelargonic)	0·67	—
Lactic	0·10	—
Pyruvic	0·01	—
Malic	0·0	—
Fumaric	0·0	—
Maleic	0·0	—
Oxalic	0·0	—
Malonic	0·0	—
Succinic	0·0	—
α-Ketoglutaric	0·0	—

The R_F values reported by Brown & Hall (1950) for fatty-acid anions, put on the chromatogram in the form of their sodium salts, are similar to those observed by us for ammonium salts. On the contrary, we have observed consistently lower R_F values when mixtures of sodium salts are used, e.g. acetic acid 0·08, propionic acid 0·12–0·14, butyric acid 0·21–0·26, *n*-valeric acid 0·37 and hexanoic acid 0·53. The reasons for this discrepancy are not clear.

Quantitative analysis

Before adopting a method involving calculation of acid content from spot area, we investigated a number of other possible techniques:

(*a*) *Titration of eluted acids.* The efficiency of a titration technique depends upon the neutralization of ammonia adsorbed on the filter paper during the development of the chromatogram, on quantitative elution of the ammonium salts from the paper and on their conversion into free acids. We found that the salts could, apparently, be eluted with distilled water and that the removal of ammonia was accomplished through the formation of hexamethylenetetramine in the presence of formaldehyde. The position of the ammonium salts at any time during development of the chromatogram could be accurately predicted from known R_F values. After 18 hr. development, the chromatogram was removed from the solvent and 1 in. (2·5 cm.) squares of paper, in the predicted position of each acid, were cut out immediately and placed in 5 ml. distilled water. After 5 min., 5 ml. of a 1:5 (v/v) aqueous dilution of B.P. formalin (exactly neutralized) were added and the free fatty acids were then titrated.

Recoveries, although not complete, showed a regular trend which was dependent on the amount of fatty acid present. It was apparent that analyses would have to be based on a standard recovery curve obtained with standard solutions run on the same chromatogram, and that this curve would vary from sheet to sheet. The technique was finally abandoned.

(*b*) *Determination of ammonia.* An indirect method based on the determination of the ammonia-nitrogen content of the ammonium salts, by reaction with Nessler's reagent after elution from paper, was also rejected. Ammonia adsorbed on the paper during development of the chromatogram caused high blanks, even after drying the chromatogram in a hot-air oven. These blanks varied from point to point on the paper, being highest near the bottom of the chromatogram.

(*c*) *Measurements of electrical conductivity.* These were made on aqueous solutions of the eluted salts, but also proved unsatisfactory.

(*d*) *Spot-area measurements—incorporation of indicator in mobile phase.* In our earlier experiments, when it appeared that variations in spraying intensity over the chromatogram affected the validity of spot-area measurements, we tried incorporating indicators in the mobile phase. With most indicators tested, exposure of the chromatogram to air after development was accompanied by the spontaneous appearance of acid spots, thus obviating the necessity for spraying with formaldehyde. The rate of appearance and stability of the spots depended largely on the pH range of the indicator, i.e. on the rate of colour change as ammonia evaporated from the paper. Indicators with a pH range within the limits 4·4–8·0 gave well defined spots which did not fade rapidly, e.g. bromocresol purple, phenol red, chlorophenol red, rosolic acid, neutral red and methyl red.

The principal objection to this technique is the fact that the R_F values of most indicators in this solvent are too low (Lederer, 1950). For instance, in phenol red ($R_F = 0{\cdot}20$) ammonium acetate appeared as a bright-yellow spot on a red background, but ammonium propionate and salts of higher acids were not covered. In rosolic acid ($R_F = 0{\cdot}40$) only ammonium acetate, propionate and butyrate could be detected as yellow spots on an orange background.

Of the indicators tested, neutral red ($R_F = 0{\cdot}66$) and methyl red ($R_F = 0{\cdot}59$) alone were satisfactory. In neutral red (0·05% (w/v) in mobile phase), magenta spots were visible on an orange background after 5–8 min. exposure to air. In methyl red (0·05% (w/v) in mobile phase), red spots appeared on a yellow background after 2–3 min. Acids from C_2 to C_6 were thus detectable, but the spots were not sufficiently well defined to allow accurate calculation of their area.

(*e*) *Spot-area measurements after spraying with bromocresol purple.* This technique was adopted only after exhaustive studies of those already outlined. Spot-area measurements may give highly inaccurate results if subsequent calculations of acid content are made from a single standard regression line obtained independently. The extent of inaccuracy depends primarily upon variations in intensity of spraying from chromatogram to chromatogram. The order of accuracy is increased enormously by chromatographing two standard solutions simultaneously with each unknown fatty-acid solution, and by calculation from the means of quadruplicate spot-area measurements.

The validity of this technique has been tested by two independent observers—one of us (R.L.R.) and another worker in this laboratory, who had previously had only a few days' experience with the technique. In each instance 5 μl. of each of six dilutions of a standard solution were run on each of four sheets, being placed on each sheet in random order to minimize bias in tracing. After development, the chromatograms were sprayed, spots traced and areas measured in the usual way. Differences between sheets were statistically significant for all acids of both observers. Calculation of the regression of spot area on logarithm of acid content from the data of the last experiment carried out by each observer gave regression coefficients which are included, together with their standard errors, in Table 2.

Table 2. *Regression of spot area on logarithm to base* 10 *of acid content* (μg./5 μl.)

(S_b is standard error of regression coefficient b.)

	Observer *A*		Observer *B*	
Acid	Regression coefficient (b) (sq.in.)	S_b	Regression coefficient (b) (sq.in.)	S_b
Acetic	+0·576	0·056	+0·458	0·064
Propionic	+0·631	0·046	+0·465	0·099
n-Butyric	+0·590	0·069	+0·599	0·114
*iso*Valeric	+0·562	0·068	+0·810	0·197
n-Hexanoic	+0·565	0·075	—	—
n-Heptanoic	+0·693	0·053	—	—

three standards on each sheet as a routine procedure (Table 3).

Errors calculated similarly from the means of triplicate estimates were no higher for the first observer, but were higher for the second observer (see Table 2).

It should be noted that the ranges of concentration given in Table 3 do not define the limits between which the relationship between spot area and logarithm of acid content holds, as the standard solutions are not included. Table 4 shows the minimum amounts of each acid which we were able to detect qualitatively and estimate quantitatively by this method.

Table 3. *Deviations of predicted from actual acid concentrations*

		Two standards on each sheet				Three standards on each sheet			
Acid	Range of concn. (μg./5μl.)	No. of concn.	Mean deviation (μg./5μl.)	Standard deviation (μg./5μl.)	Error at mid-point of range (%)	No. of concn.	Mean deviation (μg./5μl.)	Standard deviation (μg./5μl.)	Error at mid-point of range (%)
Acetic	34–93	8	−1·56	2·74	4·3	6	−2·77	2·88	4·5
Propionic	16–77	8	−1·46	1·71	3·7	6	−0·77	1·71	3·7
n-Butyric	16–63	7	+0·10	0·88	2·2	5	+0·04	0·82	2·1
n-Valeric	17–57	6	−1·18	1·16	3·2	4	−0·90	1·23	3·3
n-Hexanoic	31–71	7	−2·00	2·09	4·1	5	−0·66	1·58	3·1
n-Heptanoic	34–69	4	−1·85	1·55	3·0	3	−1·00	1·03	2·0

In each experiment the greatest and least dilutions were then treated as standards to fit a regression line from which the acid concentrations in the intermediate dilutions were predicted. In all experiments, the deviations of predicted from actual acid concentrations were independent of concentration over the range studied. Mean deviations and standard deviations calculated from the data of the first observer are given in Table 3. At the midpoints of the ranges of concentration studied, the standard deviations represent an error of 2–5 % for each acid. The errors calculated from the data obtained by the second observer were similar.

A partial explanation of the persistently negative mean differences appears to be that tracings of the small spots given by the lower standards (in which the acid concentrations were below the ranges given in Table 3) tended to give areas slightly larger than the theoretical. This personal error was most pronounced for higher acids, where the higher R_F values resulted in a more diffuse margin around the spots given by low concentrations of these acids.

Because of this, the effect on prediction errors of using three standards on each sheet was calculated from the same data, with an intermediate dilution in each series as the third standard. Mean deviations of predicted from actual concentrations were lower for each acid except acetic, but the effect on percentage error was not such as to warrant the use of

Table 4. *Minimum concentrations of acids required for detection and for quantitative estimation*

Acid	For detection (μg./5 μl.)	For quantitative estimation (μg./5 μl.)
Acetic	3	10
Propionic	3	11
n-Butyric	4	11
n-Valeric	6	13
n-Hexanoic	6	13
n-Heptanoic	7	14

Influence of some experimental conditions

Filter paper. Whatman no. 2 paper (untreated) was satisfactory for qualitative analysis, but trailing of acids, which interfered with quantitative analysis, was often observed. This trailing was not observed on Whatman no. 1 (special chromatographic) paper.

Volume of sample. Delivery of 5 μl. portions from a microsyringe is unnecessarily tedious. Micropipettes, made from calibrated thermometer tubing, are accurate and convenient. Alternatively, a haemocytometer pipette can be used. The actual volume of solution taken for analysis is, within limits, immaterial provided that the same volume is used for both standard and unknown solutions. For this reason, an appropriate calibration mark on a micropipette can be chosen to deliver a volume of

approx. 5 μl. and this volume then adhered to throughout the analysis.

Unsuitability of sodium salts. Solutions of sodium salts of fatty acids were unsatisfactory, both for qualitative and for quantitative analysis. The concentration of salts in the solution put on the chromatogram should be moderately high, otherwise acids which constitute a small fraction of the total may not be detectable in a qualitative analysis or measurable in a quantitative analysis (e.g. valeric acid in rumen liquor). Under these conditions, when relatively large amounts of sodium are present, the sodium spot overlaps or obliterates the acetate spot and prevents its quantitative determination. On several occasions we were unable to detect acetate, even though present in appreciable amounts. Acetate could be detected only when the total fatty acid content, and hence the content of Na^+, was small. This is a serious limitation in both qualitative and quantitative analysis.

Loss of acids before development. Loss of acids through evaporation may occur during the time taken to put eight or nine 5 μl. samples on to the chromatogram, particularly at higher room temperatures (25°). This loss is presumably due to displacement of fatty-acid anions from their combination with NH_4^+ by stronger acid radicals in the filter paper. It can be completely prevented by exposing the dry paper to an atmosphere saturated by aqueous 15N-ammonia for several hours before use. Similar losses, of course, do not occur during exposure of the developed chromatogram.

Measurement of spot area. The use of a planimeter gave errors as high as 40 %, an observation which appears to be contrary to the experience of Fisher *et al.* (1948) with amino-acids and sugars, but which has been confirmed by Bryant & Overell (1951). As stated elsewhere, graph-paper tracings proved entirely satisfactory and convenient. Alternative satisfactory methods include the expression of spot area in terms of the mass of the filter paper enclosed by the spot (Bryant & Overell, 1951).

Applications of the method

Lower fatty acids in rumen contents of sheep. In our experience, if lactic acid is not present, a single steam distillation in the presence of an equal volume of acidified magnesium sulphate solution (McAnally, 1944) is sufficient to effect preliminary separation of the acids. The volume of rumen liquor taken should be 2–5 ml., depending on fatty-acid concentration. Total fatty acids are determined by titration of the distillate with barium hydroxide solution of suitable concentration in the absence of carbon dioxide, phenol red being used as indicator. If lactic acid is present, double steam distillation can be carried out by the method of Friedemann (1938) although, in our experience with rumen liquor containing up to 5 mg.mol. lactic acid in the presence of 10–12 mg.mol. lower fatty acids per 100 ml., the amount of lactic acid which distils over in a single distillation in the Markham apparatus causes an error of only 3–5 % in the determination of acetic acid. For many practical purposes this error can be neglected, thus avoiding the necessity for double distillation.

After addition of excess barium hydroxide, the first 30 ml. of distillate is evaporated to dryness, the later stages of evaporation being carried out after transfer of the solution to a 10 ml., or smaller, centrifuge tube. Conversion of the barium salts to ammonium salts is effected by addition of a suitable known volume of saturated ammonium oxalate solution (usually 0·5–1·0 ml.). The concentration of ammonium salts in the final solution obviously depends on the solubility of ammonium oxalate, and is of the order of 2·0–2·5 μg.mol./5 μl.

After centrifuging to remove barium oxalate, 5 μl. portions of the supernatant solution, containing the ammonium salts of the fatty acids, together with excess ammonium oxalate, are transferred to paper for the subsequent analysis. Ammonium oxalate remains on the 'starting line' (cf. Table 1) and the presence of an oxalic acid spot in this position after spraying provides a check as to whether sufficient ammonium oxalate was originally added to precipitate all barium. Phenol red does not interfere with the analysis and usually moves to a position between ammonium acetate and ammonium propionate.

The percentage composition of the mixture of lower fatty acids in rumen liquor is remarkably constant under a wide range of dietary conditions, which facilitates the choosing of suitable standard solutions. As C_5 acids constitute only 3–6 % of the fatty acids present, the amounts run on the chromatogram (6–12 μg.) are outside the range of the method. It is possible, however, to make a reasonably accurate estimate by direct visual comparison with suitable standards (6 and 12 μg./5 μl.). The amounts of C_6 acid present are too small to be detected by this method.

Several analyses, both by this method and by that of Moyle *et al.* (1948), have shown that although the latter method is the more accurate, results by the two methods are, consistently, in reasonable agreement (Table 5).

To date we have made seventy-two analyses of rumen liquor. Recoveries in sixty-six of these, based on the total acid titration of the first distillate, ranged from 91 to 108 %, with a mean of 97·4 ± 4·2%. Recoveries would not be expected to exceed this value, as small amounts of acids higher than C_5 are not detected.

Fatty-acid metabolism in nematode parasites. The method has been used successfully by another

Table 5. *Comparison of present method with that of Moyle, Baldwin & Scarisbrick* (1948)

(Acids as mmol./100 ml. rumen liquor.)

Rumen liquor sample no.	Present method					Method of Moyle *et al.*			
	C_2	C_3	C_4	C_5	Total recovery (%)	C_2	C_3	$C_4^{\dagger}$	Total recovery (%)
1	7·38	2·28	1·56	0·90	94	7·29	2·38	2·48	94
2	7·80	2·40	1·68	0·90	93	8·19	2·41	2·82	97
3	7·92	1·80	1·74	0·90	94	7·89	2·16	2·67	97
4	4·63	0·90	0·86	0·48	95·6	4·73	0·89	1·43	99·5
5	6·60	1·80	1·39	0·51	97·5	6·69	1·75	1·91	98
6	6·95	1·95	1·46	0·54	98	7·12	1·99	2·12	101

worker in this laboratory as a convenient approach to metabolic studies, involving fatty acids as high as hexanoic, in nematodes. Preliminary separation of the acids from homogenized tissue extracts by the method of Friedemann (1938), or by distillation as described by McClendon (1944), is followed by the same general procedure outlined herein for analyses of rumen liquor, but suitably modified to cope with the much smaller quantities of material available. Recoveries have been of the order of 95–102 %.

Limitations of the method

The principal limitations of the method have already been discussed. It is less accurate than that of Moyle *et al.* (1948), and isomeric acids and acids higher than C_7 cannot be separated. Nevertheless, in our hands, the method has proved considerably more convenient than its predecessors and, as any inaccuracy involved has usually been within the limits of normal biological variation, it is of little consequence.

The failure to separate acids higher than C_7 is not an intrinsic limitation to paper partition-chromatographic separation of these acids. We have observed considerably lower R_F values in pentanol, which suggests that higher fatty acids may be separable in higher-alcohol solvents. Isomeric acids have not so far been separated.

SUMMARY

1. A method is described by which steam-volatile saturated fatty acids can be separated, identified and estimated by paper-partition chromatography.

2. The acids are run upward on paper as their ammonium salts using *n*-butanol-aqueous ammonia as solvent system. The free fatty acids appear as yellow spots on a purple background after the developed chromatogram is sprayed with a bromocresol purple solution containing formaldehyde. Fatty-acid content can be calculated from spot area because of the direct relationship between spot area and the logarithm of acid content.

3. Fatty acids of the normal series from acetic to heptanoic can be separated, identified and estimated with an accuracy of ±2–5 % for each acid. Heptanoic acid cannot be separated from octanoic and nonanoic acids; octanoic acid can only be estimated in the absence of heptanoic and nonanoic acids. Isomeric acids cannot be separated.

We with to express our sincere appreciation to Miss M. C. McKevett, who carried out the numerous statistical analyses essential to the development of the method.

Our thanks are also due to Dr W. P. Rogers, Miss H. N. Turner, Mr J. Hogan and Mr W. K. Warburton for their assistance at various stages of this work. We are also indebted to Mr G. K Hughes for gifts of fatty acids.

Note. Since this manuscript was completed, a recent paper by Hiscox & Berridge (1950) has come to hand. These authors have separated volatile fatty acids on paper in a mobile phase of butanol, with ethylamine in the vapour phase. After spraying with bromocresol green they observed that the 'area of the spot gives a rough indication of the total quantity of fatty acid present' and have used the method to obtain 'roughly quantitative' results.

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The Relative Rates of Oxidation of Lipovitellin and of its Lipid Constituent

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Unsaturated lipids in general, and phospholipins in particular, are known to undergo oxidation when exposed to the atmosphere, and such changes are liable to occur whenever lipid-containing substances are handled. The recent observation by Dam and co-workers (Dam, 1944 *a*, *b*; Dam & Granados, 1945 *a*, *b*) that fat peroxides and a yellow-brown discoloration can develop in tissues *in vivo*, particularly when the diet is deficient in tocopherols (which act as anti-oxidants) and rich in highly unsaturated fatty acids, lends added biochemical interest to a process hitherto considered mainly of importance in extracted lipids and 'dead' tissues.

Lipids, which in the isolated state are very susceptible to attack by oxygen, often show in the tissue an astonishing resistance; linseed oil, for example, remains unoxidized for long periods in the harvested flax-seed. Although the difference is probably connected with the fact that inhibiting substances such as phenolic anti-oxidants are only in part extracted with the lipid, the further possibility exists that the more reactive lipids may be stabilized against oxidation by existing in combination with other constituents of the tissues. Triglyceride fat, and particularly that present in the large fat storage depots, is mainly free, whilst much of the phospholipin occurs in combination with protein or (in less degree) carbohydrate in the cells. It seemed of interest, therefore, to compare the rates of oxidation, both in the presence and absence of catalysts, of phospholipin (*a*) in combination with protein, and (*b*) in the free state, either alone or admixed with the protein.

Lipoproteins in general tend to be ill defined, non-crystalline, labile substances, difficult to isolate, characterize and handle. Lipovitellin from egg yolk was chosen for the present work because its comparatively high stability and low degree of unsaturation favoured separation in an undecomposed and unoxidized state. Since the autoxidation of lipids can be greatly accelerated by exposure to light or by the presence of trace metal and other contaminants, or retarded by substances which function as anti-oxidants (inhibitors), the technique employed included precautions against interference by such factors.

EXPERIMENTAL

Preparation of lipovitellin from egg yolk

The method used was essentially that of Chargaff (1942), with modification where necessary to overcome the intractable emulsions encountered in the original procedure. Batches of 48 eggs, not more than 24 hr. old, were washed in 2% lysol and in ethanol, which was burned off before breaking. The yolks, after removal of the membrane and chalazae, were mixed with an equal volume of water and extracted by shaking five or six times with 800 ml. peroxide-free ether until the extract was practically colourless. Saturated NaCl solution (520 ml.) was then added to dissolve the lipoprotein, and the solution was dialysed through cellulose, first into water (16 l.) for several hours and then overnight into water (80 l.). All operations were carried out at 0–3° with precooled solvents and equipment. The very large quantities of water required for dialysis were prepared by mixed bed de-ionization of the laboratory distilled water supply. Cellulose casing was found to contain considerable quantities of water-soluble material and was thoroughly extracted before use.

The crude lipoprotein which separated was centrifuged, washed twice with 800 ml. water, suspended in 560 ml. water and shaken with 800 ml. ether. No ether could be separated at this stage, and 240 ml. saturated NaCl solution were added and the whole dialysed. During dialysis an ether layer separated and was discarded before centrifuging the protein. The processes of solution by NaCl, reprecipitation by dialysis, washing and extraction with ether were repeated four times, with omission of the ether extraction on the last occasion. The lipoprotein suspension obtained was held at 0° in an atmosphere of N_2, and its solids content determined by drying to constant weight *in vacuo* at 70°.

Fevold & Lausten (1946), by direct centrifugation of diluted egg yolk in a Sharples centrifuge at 40,000 rev./min., separated a solid crude 'lipovitellin' fraction which, after purification by Chargaff's method, contained 16–18% lipid. From the residual emulsion by a similar purification they recovered a product of higher lipid content (36–41%) which they termed 'lipovitellinin'. In our hands separation by the Sharples centrifuge at 24,000 rev./min. (the high-speed turbine head was not available) gave a lipovitellin fraction containing only about 13% of lipid. It therefore seems probable that lipoproteins of egg yolk range widely in fat content, and 'lipovitellin' and 'lipovitellinin' are probably not well defined entities. The lipoprotein obtained by the modified Chargaff procedure described above contained 22–30% of lipid and was considered suitable for the present work.

The ethanol-soluble lipid content of lipovitellin. In preliminary attempts to determine the total lipid content of the aqueous lipovitellin cream by extraction with ethanol and ether, before or after ordinary drying, trouble was encountered with emulsification or incomplete extraction. In the method finally adopted, the lipovitellin suspension (approx. 10% solids) was freeze-dried and extracted in a Soxhlet apparatus with hot ethanol for 4 hr. The extract was evaporated in a rotary film vacuum evaporator (Lea, Hannan & Rhodes, 1951), taken up in dry ether, filtered, evaporated under an infrared lamp and dried in a vacuum oven at 70°. Typical results, compared with those obtained by the repeated cold extraction procedure used by Fevold & Lausten, are given in Table 1.

Table 1. *Determination of total lipid content of lipovitellin*

Temp. of ethanol (°)	Time of extraction in Soxhlet apparatus (hr.)	No. of separate 30 min. extractions	Lipid extracted (dry wt. basis) (%)
75	2	—	25·0
75	4	—	25·7
75	6	—	25·7
Approx. 20	—	1	19·7
Approx. 20	—	2	22·1
Approx. 20	—	7	25·2

Determination of free lipid. For the estimation of free lipid present as a contaminant or produced by decomposition of the lipoprotein, it was necessary to extract the aqueous lipoprotein solution or suspension with ether. Under most conditions this gave rise to intractable emulsions, but by extracting 3 ml. of a solution containing approx. 0·5 g. lipoprotein and 0·3 g. NaCl with successive portions (100 ml.) of ether saturated with water, a satisfactory separation was achieved. 'Wrist-action' agitation in a 'Microid' shaker served to mix the phases.

When free lipid was present, it was very largely extracted in 10–30 min. at 0–3°, but prolonged extraction in this way showed that the lipovitellin preparations yielded virtually no free lipid until it slowly became soluble owing to decomposition or denaturation (Fig. 1).

Splitting with ethanol

For comparison of the rate of autoxidation of lipid combined in the lipoprotein with that of the free lipid in presence of the protein, it was necessary to split the lipoprotein complex with minimal damage to either component, and then to remove completely any agent used for splitting. This was first attempted *in situ* without gross separation of the two components, by freeze-drying the lipovitellin suspension (4 g. containing approx. 0·5 g. dry material), just moistening with absolute ethanol (2 ml.), holding for 4 hr. at the selected temperature and removing the ethanol by vacuum evaporation at the same or a lower temperature. Residual traces of ethanol were removed by addition of water and freeze-drying. Treatment with ethanol at 25° or below was found to render less than half the total lipid extractable with ether, and even ethanol at 75° failed to liberate more than 60% of the lipid (Fig. 1). Apparently, mechanical removal of the lipid as it is liberated is essential for complete splitting of the lipoprotein complex by ethanol.

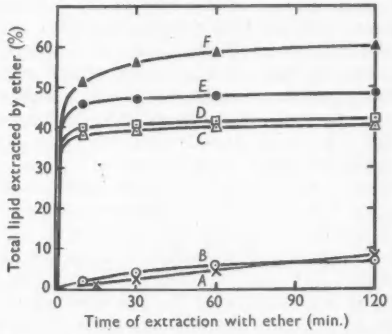

Fig. 1. Effect of ethanol at various temperatures on the stability of lipovitellin. *A*, undried; *B*, freeze-dried; *C*, dried and treated with ethanol at −7°; *D*, dried and treated with ethanol at 10°; *E*, dried and treated with ethanol at 25°; *F*, dried and treated with ethanol at 75°.

Separation of the lipid from the protein. The efficiency of solvent extraction at temperatures between −28° and +25° was investigated by repeatedly extracting (with shaking) freeze-dried lipovitellin with ethanol or ethanol-ether,

Table 2. *Extraction of lipid from freeze-dried lipovitellin by solvents*

Lipid in the lipoprotein (%)	Solvent	Temp. (°)	No. of 30 min. extractions	Percentage of total lipid extracted
29·1	Ethanol-ether, 1:1 (v/v)	− 7	5	76·8
29·1	Ethanol	−28	5*	77·4
25·7	Ethanol	− 7	4*	88·3
25·7	Ethanol	+25	4*	97·9

* Followed by two extractions with ether.

followed by evaporation of the solvent in a rotary film vacuum evaporator at <40°. In each case ethanol was thoroughly removed from the protein before the temperature was allowed to rise, by drawing cold air through the protein on a sintered-glass filter for 1 hr., followed by two 30 min. extractions with ether and further aeration for 1 hr. Hewitt (1927) has pointed out that such thorough removal of ethanol is necessary if the denaturation of plasma proteins is to be prevented. Since extraction of the lipid was far from complete at −28° (Table 2), whilst at 25° the protein would certainly be damaged, extraction with ethanol at −7° (followed by ether as described above) was adopted as a compromise.

Emulsification of the lipid and of the lipid-protein mixture. Notwithstanding the known properties of phospholipins as emulsifying agents for oil-water systems, the dispersal of the free lipid in water for oxidation studies proved to be a matter of some difficulty. Two small impulse-type homogenizers tested had working parts of plated copper alloy and gave excellent emulsions, but enormously accelerated oxidation owing to metallic contamination. Replacement of the piston and cylinder by stainless steel resulted in rapid abrasion and consequent contamination. The all-glass apparatus finally employed was constructed from the lower half of a Pyrex glass boiling tube fused to a glass rod and rotated inside a slightly larger glass tube by a motor with a flexible coupling. After 'running in' with a little emery paste the fit of the tubes was such that the outer dropped only very slowly (or not at all) from the inner under its own weight. In operation the outer tube was slowly raised and lowered, thus pumping the fluid through the gap between rotor and stator. The apparatus was used in a room at 0–3° to minimize heating effects. Emulsions obtained in this way were neither so fine nor so uniform as those obtainable with a metal homogenizer or colloid mill, but were uncontaminated and oxidized at reasonably reproducible rates.

Free lipid in the lipid-protein mixture. When the lipid and protein constituents of lipovitellin, separated by extraction of the freeze-dried lipoprotein with ethanol and ether at −7° or at +25°, were re-emulsified together in water and the free lipid content of the mixture determined, it was found that not all of the added lipid could be re-extracted with ether. Thus, from a specimen of lipovitellin containing 25·7% of total lipid, 22·7% lipid was separated by repeated extraction with ethanol and ether at −7°. After re-emulsification of this lipid with the protein, however, only 20·6% lipid could be recovered by prolonged extraction with ether. In a second experiment 25·2% lipid was separated by extraction with ethanol and ether at 25°, but after re-emulsification with the protein only 22·1% could be recovered by ether extraction.

While these observations do indicate a certain degree of holding or binding of the lipid by the protein in the presence of water, they do not constitute proof of the regeneration of any part of the original lipovitellin complex.

Measurement of absorption of oxygen

The stock lipovitellin suspension was diluted to contain 100 mg. lipid/3·5 g. and this quantity, as delivered by a calibrated all-glass syringe, was placed in a Warburg flask together with (usually) 0·5 ml. 0·1 M-phosphate or 0·25 M-acetate, with 0·3 ml. catalyst solution in the side bulb and water to a total of 5 ml. in the flask. The buffers had previously been adjusted by titration with the glass electrode in presence of the required concentration of lipoprotein and salt to give a final pH of 6·8 or 5·2 respectively. 0·8% NaCl was usually added, to bring the total ionic strength into the physiological region. KOH was used in the centre cups, and an atmosphere of O_2 in the flasks. The top of the bath was partly covered, and the water dyed dark red to prevent acceleration of the oxidation by the ultraviolet and blue components of daylight.

Since runs were usually continued for 30 hr. at 40°, precautions had to be taken against microbial growth, despite the somewhat unfavourable nature of the medium and low initial counts. Increasing the temperature to 45° reduced bacterial growth, but increased decomposition of the lipoprotein. Experiments were therefore carried out in duplicate at 40° with replacement of the NaCl by NaF. Typical 37° counts/ml. after 48 hr. at 40° were $>10^6$ with chloride, <10 with fluoride at pH 6·8, and 20–30 with chloride, <10 with fluoride at pH 5·2. Decomposition of the lipovitellin during 30 hr. as measured by the liberation of free lipid was of the order of 4% of the total lipid at pH 6·8 and 8% at pH 5·2. The effects of the buffer salts and of chloride and fluoride on the rate of oxidation were investigated separately.

Catalysts. Since tissues in which lipoproteins or phospholipins are present frequently also contain known catalysts for the oxidation of unsaturated fatty acids, comparisons of the rate of oxidation of lipovitellin with that of its lipid constituent were also carried out in the presence of haemoglobin, of cytochrome *c* and of haemin. The effect of ionic copper, which is liable to be present in food or in biochemical preparations as a contaminant, was also investigated.

Haemoglobin. Oxyhaemoglobin was prepared as required from a concentrated solution of purified ox haemoglobin preserved as the CO compound at −20° and diluted to contain 5 μg. iron/0·3 ml.

Cytochrome c. Stock solutions of horse-heart preparations (Keilin & Hartree, 1945) were diluted to contain 5 μg. iron/0·3 ml.

Haemin. Haemin (ferrihaem chloride) was dissolved in dilute NaOH and diluted to contain 5 μg. iron/0·3 ml. Acid equivalent to the alkali used was added to the Warburg flask.

Copper. A slightly acidified $CuSO_4$ solution was diluted to contain 5 or 1 μg. Cu/0·3 ml. Alkali equivalent to the acid used was added to the Warburg flask.

RESULTS

Effect of pH *and of phosphate, acetate, chloride and fluoride on the rate of oxidation*

Lipovitellin. Uncatalysed lipovitellin at pH 6·8 or 5·2 absorbed oxygen at an approximately linear rate over long periods. Neither phosphate nor acetate at the concentrations used had any appreciable effect on the rate of oxidation (Table 3). Sodium chloride (0·8%, w/v) nearly doubled the rate of oxidation at pH 5·2, although at pH 6·8 it was without effect. Fluoride had no effect at either pH. Decreasing the pH from 6·8 to 5·2 increased the rate of oxidation by 50–60% in the absence of added chloride, and approximately trebled the rate in its presence.

Table 3. *Effect of* pH, *chloride, fluoride, phosphate and acetate on the rate of oxidation* of lipovitellin*

Buffer	Salt (%, w/v)	Lipovitellin preparation no. 6	10	11	12	Average	Ratio: pH 5·2 / pH 6·8
		pH 6·8					
—†	—	1·1	0·9	1·1	1·2	**1·1**	—
0·01 M-Phosphate	—	1·0	1·0	1·0	1·0	**1·0**	—
0·01 M-Phosphate	NaCl, 0·8	0·8	0·7	0·8	0·9	**0·8**	—
0·01 M-Phosphate	NaF, 0·8	1·1	0·8	0·8	1·0	**0·9**	—
		pH 5·2					
—†	—	1·3	2·0	1·7	2·3	**1·8**	1·6
0·025 M-Acetate	—	1·4	1·4	1·6	1·7	**1·5**	1·5
0·025 M-Acetate	NaCl, 0·8	2·3	2·8	2·3	3·3	**2·7**	3·4
0·025 M-Acetate	NaF, 0·8	1·2	1·4	1·4	1·8	**1·5**	1·6

* Average rate of oxidation, period 0–30 hr., based on rate in phosphate buffer without salt as 1·0 (actual average rate under these conditions = 6·9 μl. O_2/100 mg. lipid/hr.).
† Self buffered; little or no pH change occurred.

Table 4. *Effect of* pH, *chloride, fluoride, phosphate and acetate on the rate of oxidation* of 'split' lipovitellin*

Buffer	Salt (%, w/v)	Lipovitellin preparation no. 10	11	12	Average	Ratio: pH 5·2 / pH 6·8
		pH 6·8				
—†	—	1·0	1·0	1·0	**1·0**	—
0·01 M-Phosphate	—	1·0	1·0	1·0	**1·0**	—
0·01 M-Phosphate	NaCl, 0·8	0·8	0·8	0·9	**0·8**	—
0·01 M-Phosphate	NaF, 0·8	1·1	1·4	1·3	**1·3**	—
		pH 5·2				
—†	—	1·1	1·1	1·0	**1·1**	1·1
0·025 M-Acetate	—	0·9	1·4	1·5	**1·3**	1·3
0·025 M-Acetate	NaCl, 0·8	1·1	1·8	1·6	**1·5**	1·9
0·025 M-Acetate	NaF, 0·8	1·1	1·8	1·7	**1·5**	1·2

* Average rate of oxidation, period 0–30 hr., based on rate in phosphate buffer without salt as 1·0 (actual average rate under these conditions = 3·3 μl. O_2/100 mg. lipid/hr.).
† Self buffered; little or no pH change occurred.

Further investigation of the influence of sodium chloride on the oxidation of lipovitellin showed that at pH 5·2 the maximum acceleration appeared to be reached at a concentration in the neighbourhood of 0·8 %, whilst higher concentrations of chloride produced a gradual decrease in rate. At pH 6·8 increasing concentrations of chloride produced a gradual decrease in rate without the initial acceleration (Fig. 2). Oxidation of lipovitellin at pH 5·2 in the presence of 0·8 % sodium chloride in (*a*) 100 % oxygen, (*b*) 88 % oxygen, 12 % nitrogen, and (*c*) 75 % oxygen, 25 % nitrogen (the latter two atmospheres corresponding approximately to the reduced solubility of oxygen produced by increasing the salt concentration from 0·8 to 3 and 6 % (w/v) respectively), however, indicated that the falling off in rate of oxidation with increasing concentration of sodium chloride was probably due to the decreasing solubility of oxygen under these conditions. The direct effect of sodium chloride at pH 5·2 therefore appears to reach a maximum at 1 % or below, and thereafter to remain constant up to 6 % at least. This salt, apparently, had little or no direct effect on the rate of oxidation at pH 6·8.

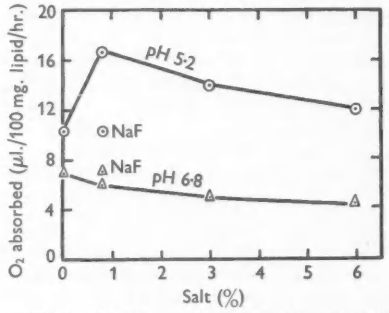

Fig. 2. Effect of 0–6% sodium chloride and of 0·8% sodium fluoride on the oxidation of lipovitellin in buffer at pH 5·2 and 6·8.

'Split' lipovitellin. Neither phosphate nor acetate, chloride nor fluoride had any marked effect on the rate of oxidation of the lipid-protein mixture produced by splitting off the lipid from lipovitellin and subsequently re-emulsifying it with the protein. Oxidation tended to be slightly faster at pH 5·2 than at pH 6·8, particularly in the presence of sodium chloride (Table 4).

Lipid. Oxidations of the emulsified free lipid were carried out only in the presence of buffer. Little, if any, effect of chloride or fluoride was apparent, and the influence of pH, over the limited range covered, appeared to be small.

Relative rates of oxidation of lipovitellin and of 'split' lipovitellin (uncatalysed reaction)

The rates of absorption of oxygen by four preparations of lipovitellin, and by the same preparations after splitting off the lipid with ethanol at −7° and re-emulsifying with the protein, as described above, are compared in Fig. 3. In every case

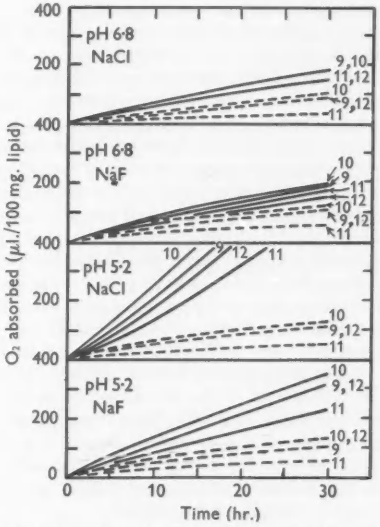

Fig. 3. Relative rates of oxidation of lipovitellin (——) and of 'split' lipovitellin (– – –) in buffered suspension in the presence of chloride or fluoride. Preparations 9–12.

the lipoprotein oxidized more rapidly than the split product. At pH 6·8 the ratio of rates was rather less than 2:1; at pH 5·2 the ratio was about 2·5:1 in the presence of fluoride and over 3:1 in the presence of chloride.

Influence of catalysts in the three systems

The effects of copper, haemin, haemoglobin and cytochrome *c* on the oxidation of lipovitellin, of 'split' lipovitellin and of the free lipid are shown in Figs. 4–7.

Copper. Copper greatly accelerated the oxidation of the free lipid at both pH values (Fig. 4). The presence of protein in the system, whether free or bound to the lipid, greatly reduced the accelerating action of the copper and, in the case of the 'split' lipovitellin, eliminated it almost completely.

Haemin. Haemin showed powerful catalytic activity in all three systems, acceleration being greatest with the lipid and least with the lipoprotein

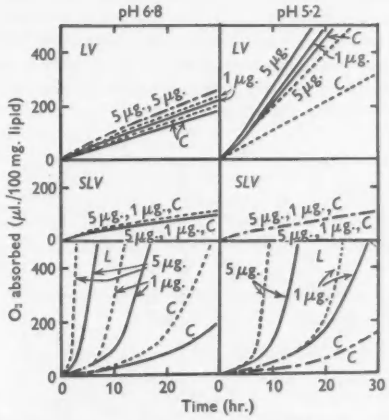

Fig. 4. Effect of copper (1 μg. and 5 μg.) on the oxidation of lipovitellin (*LV*), 'split' lipovitellin (*SLV*) and the separated lipid (*L*) at pH 6·8 and 5·2 in buffered NaCl (——) and NaF (- - - -). *C* = control.

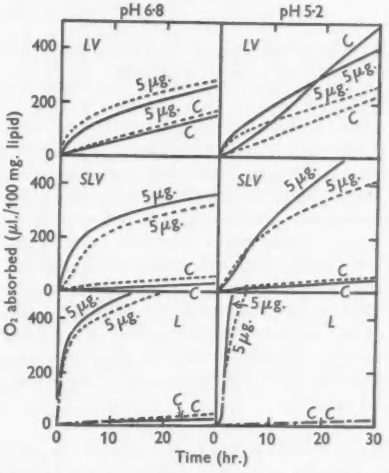

Fig. 5. Effect of haemin (5 μg. Fe) on the oxidation of lipovitellin (*LV*), 'split' lipovitellin (*SLV*) and the separated lipid (*L*) at pH 6·8 and 5·2 in buffered NaCl (——) and NaF (- - - -). *C* = control.

(Fig. 5). The shape of the oxygen absorption curves in all cases indicated a probable destruction of the catalyst as oxidation proceeded. This behaviour is in accord with the observations of Haurowitz, Schwerin & Yenson (1941), who found a destruction of haemin or haemoglobin to be coupled with the

oxidation of linoleic or linolenic acid in air at 38°, a considerable proportion of the haematin iron being liberated ultimately in the inorganic state.

Haemoglobin. Haemoglobin showed about the same accelerating activity as haemin in lipovitellin, but, in contrast to haemin, was comparatively ineffective with the free lipid (Fig. 6). The curves again provided evidence of a destruction of the catalyst as oxidation proceeded.

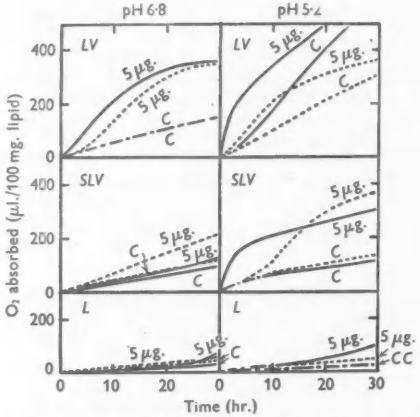

Fig. 6. Effect of haemoglobin (5 μg. Fe) on the oxidation of lipovitellin (*LV*), 'split' lipovitellin (*SLV*) and the separated lipid (*L*) at pH 6·8 and 5·2 in buffered NaCl (——) and NaF (- - -). *C* = control.

Cytochrome c. The effect of cytochrome *c* on oxidation was small and the results have not been presented in detail. With lipovitellin a slight but definite and consistent retardation was observed. With 'split' lipovitellin the effect of cytochrome *c* was negligible. With the free lipid oxidation was slightly accelerated by cytochrome *c* in the presence of chloride, particularly at the more acid pH, but the effect was small and appeared to be inhibited by fluoride. Experiments with other specimens of cytochrome at concentrations of 0·5–5 μg. iron gave similar results.

DISCUSSION

A major difficulty, which prevented a rigid comparison of the rates of oxidation of the lipoprotein complex, of the lipid-protein mixture ('split' lipoprotein) and of the isolated lipid component itself, was the impossibility of obtaining the lipid in the three systems in the same physical state, particularly with respect to degree of dispersion. Despite this handicap, the results clearly afford no support for the widely held view that lipids of lipoproteins are greatly stabilized against oxidation by oxygen. Thus, although dried plasma, if stored in the presence of oxygen, develops rancidity quite readily (Ratcliff, 1950), it has been pointed out that there is no evidence of rapid oxidation of lecithin in normal plasma (Frazer, 1949). Even with particular reference to lipovitellin, Fevold & Lausten (1946) write: 'Another fact that has become apparent in this work is the increased stability of the phospholipins when in combination with the proteins. The combination appears to be a somewhat loose one as judged by the ease of breaking. However, the typical browning of uncombined phospholipins on exposure to air takes place only very slowly in the phospholipins combined in the lipoproteins.' In the present work, when the lipid and protein fractions of lipovitellin were separated with some care, remixed, and subjected to the same carefully controlled oxidizing conditions as the original lipoprotein, no increase in rate of oxidation was observed. On the contrary, an appreciable decrease in the rate of oxidation occurred, although no particular significance has been attached to it in view of experimental limitations and possible partial denaturation of protein during separation. In certain complex food systems, e.g. milk (Findlay, Higginbottom, Smith & Lea, 1946), heating under conditions which expose protein sulphydryl groups has been found to increase the resistance of the fat present to autoxidation. Free phosphatides are normally preserved in the dry state and lipoproteins in an aqueous phase, which reduces the concentration of oxygen in contact with the lipid by about forty times for normal saline or sixty times for the 10 % sodium chloride used by Fevold & Lausten to hold lipovitellin in solution (values calculated for 30°). This may account for part at least of the observed behaviour. Lipoprotein may be completely protected from autoxidation inside tissues such as muscle, which possess an appreciable residual oxygen uptake.

Since the same general result was obtained in the presence of chloride or fluoride, the probable absence of significant interference by bacterial growth in the chloride experiments or of specific interference by fluoride could be inferred. It was observed, however, that whilst the oxidation of the 'split' mixture was not appreciably influenced by pH (6·8 or 5·2) or by the nature of the salt present (sodium chloride or fluoride), the intact lipoprotein oxidized appreciably more rapidly at the more acid pH and, at this pH, was more readily oxidizable in the presence of chloride than of fluoride (Fig. 3). The observed effect of chloride (Fig. 2) may have been somewhat too low, since no particular precautions were taken to free the lipovitellin from the last traces of the chloride used so freely in its preparation. The mechanism of the action of chloride is unlikely to be connected with the solubility of the lipoprotein, because the maximum accelerating effect was reached at salt concentrations too low seriously to affect solubility. Since activity at pH 5·2 but not at

pH 6·8, and activation by chloride but probably not by fluoride, are properties of the ill defined unsaturated fatty acid 'lipoxidase' of mammalian muscle (Lea, 1937), it is possible that contamination with such an enzyme rather than the nature of the lipoprotein itself might be responsible for the observed results. In the absence of adequate data on the properties of the 'lipoxidase', it was not considered profitable to pursue the matter further at this stage.

Concerning the effects of catalysts, combination with protein undoubtedly largely protected the lipid from the catalytic action of copper, presumably owing to the binding of a large proportion of the copper in a non-ionic form. But combination with protein was no better in this respect than simple admixture; in fact copper was inactivated even more efficiently by the free than by the combined protein (Fig. 4). This might have been due either (*a*) to copper being bound by protein groups which in turn are combined with lipid in the lipoprotein, or (*b*) to a certain degree of denaturation of the protein, with unmasking of copper-binding sulphydryl groups, during removal of the lipid from the lipoprotein.

In catalysis by haemin, the presence of protein afforded partial although not complete protection, and in this case combination with the protein was appreciably more effective than simple admixture (Fig. 5). Almost the reverse was true with haemoglobin where reactive groups of the free phosphatide appear to have inactivated the catalyst more readily in the absence of other protein.

Cytochrome *c*, found by Banks (1949) to be the most active of the natural haematin pigments for the oxidation of linoleic acid emulsions in phosphate at pH 6·6, was comparatively ineffective as a catalyst for the oxidation of the lipid (mainly phospholipin) of lipovitellin under all conditions tested.

The action of ethanol in disrupting the lipid-protein linkage is widely known and utilized in extraction procedures, although the mechanism of its action (often assumed to be one of dehydration) and even the nature of the union between lipid and protein is largely unknown. Some of the lipid, particularly the more inert fractions such as cholesterol and its esters, appears to exist as solid solution in the prosthetic portion of the lipoprotein. For this reason the action of limited quantities of ethanol at $-7°$ to $+25°$ in rendering only 40–50% of the lipid extractable with ether may be of value as a possible means of fractionating the bound lipid. In this connexion Delsal (1949) has recently observed that extraction of blood serum with ether at $-70°$ according to the procedure of McFarlane (1942) removes only cholesterol from serum, leaving the phospholipin-protein complex intact. The observation that a fraction of the lipid, although only a comparatively small one, becomes unextractable with ether when the separated lipid and protein components of lipovitellin are remixed is also of some interest.

SUMMARY

1. Lipovitellin from egg yolk has been split by treatment with ethanol at low temperature into its lipid and protein components and the rates of autoxidation at 37° of the original lipoprotein, of the remixed lipid and protein, and of the lipid alone compared at pH 6·8 and 5·2.

2. In the uncatalysed systems no support could be found for the widely held view that phospholipins are greatly stabilized against autoxidation by combination with protein.

3. Combination with protein gave considerable protection against the catalytic action of copper and of haemin, but not against the (weaker) action of haemoglobin. Copper, however, was at least as effectively inactivated by free protein as when the latter was combined with the lipid.

4. Cytochrome *c* had very little catalytic action on the oxidation of lipid of lipovitellin whether free or combined.

5. The lipovitellin preparations used autoxidized more rapidly at pH 5·2 than at pH 6·8, and at the more acid pH the rate was further accelerated by the presence of chloride but not of fluoride.

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Enzymes Concerned in the Synthesis of Glycogen from Glucose in the Brown Adipose Tissue

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Under normal conditions glycogen cannot be detected in adipose tissue. When animals are first starved and then fed on a carbohydrate diet, glycogen appears temporarily in this tissue (see Hausberger & Neuenschwander-Lemmer, 1939). Tuerkischer & Wertheimer (1942) investigated the phenomenon in detail and found that, in the rat, this deposition of glycogen was particularly marked in the brown adipose tissue, which in this species is found between the scapulae as a light-brown butterfly-shaped mass, having the appearance of a gland. Glycogen concentrations as high as 6 g./100 g. wet tissue were sometimes observed. Fawcett (1948) has recently confirmed these observations. Mirski (1942) showed that brown adipose tissue of the rat contained phosphorylase and phosphoglucomutase and suggested that glycogen might be synthesized from glucose by a similar mechanism to that involving hexokinase and these enzymes in muscle and liver.

Cori & Green (1943) and Cori & Cori (1945) showed that suitable preparations of muscle and spleen could convert crystalline muscle phosphorylase *a* into phosphorylase *b*, which was active only in the presence of adenylic acid. This effect of muscle and spleen was attributed to the presence of a 'PR' (prosthetic group-removing) enzyme, which was considered to remove a prosthetic group from phosphorylase *a*. Cori & Cori (1943) observed that the presence of extracts of liver or heart resulted in the synthesis by phosphorylase *a* from glucose-1-phosphate of a polysaccharide similar to glycogen, while in the absence of such extracts there resulted a polysaccharide similar to amylose. They believed such extracts contained a 'Supplementary Enzyme' which, while phosphorylase *a* was forming, α-1:4-glucoside linkages catalysed the introduction of branch points formed by α-1:6-glucoside linkages.

The work described in this paper has demonstrated the presence of hexokinase in the brown adipose tissue of the rat, and has confirmed the presence of phosphorylase and phosphoglucomutase; the properties of the latter two enzymes have been investigated in more detail. Brown adipose-tissue extracts have also been found to possess PR enzyme and Supplementary Enzyme activities.

EXPERIMENTAL

Reagents

Veronal acetate buffers (0·034 M) were prepared according to Gortner (1949).

Adenosinetriphosphate (ATP). The ATP used (Boots Pure Drug Co. Ltd.) was 80% pure according to its content of acid-labile P (7 min. in N-HCl at 100°). A 0·05 M-ATP solution of the Na salt was prepared from the Ba salt.

Adenylic acid was prepared from dibarium ATP by the method of Kerr (1941).

Glucose-1-phosphate was prepared enzymically from potato starch by a modification of the method of Hanes (1940).

Cysteine HCl was obtained from Lights Ltd. Sodium β-glycerophosphate, sodium fluoride, α-methyl-D-glucoside and phlorrhizin were British Drug Houses (B.D.H.) Laboratory grade. All other chemicals used were B.D.H. Analar grade.

Aluminium hydroxide C_γ suspensions were prepared by the method of Bertho & Grassmann (1938).

Crystalline phosphorylase a. Suspensions were prepared according to Cori, Cori & Green (1943).

Glycogen was prepared from rabbit liver by the method of Somogyi (1934).

Analytical methods

Glucose was determined by the photometric method of Nelson (1944) using the Somogyi (1945) copper reagent.

Inorganic phosphorus was determined according to Fiske & Subbarow (1925) modified by Umbreit, Burris & Stauffer (1945).

Glycogen was determined by the method of Colowick & Sutherland (1942).

Preparations of adipose tissue extracts

Wistar rats were used throughout. All extracts investigated for enzyme activity were prepared as quickly as possible after killing the animal by a blow on the head. The brown adipose tissue is supported between the scapulae by strands of muscle which would contain the enzymes being sought in the tissue; particular care was therefore taken to remove all of this muscle before preparing the extracts. The brown adipose tissue was freed from white adipose tissue and stored on ice until sufficient material had been dissected out. After the fat had solidified it became much lighter in colour, and any remaining muscle could be distinguished as darker, translucent material which was easily removed. All subsequent operations were carried out in the cold room at 5° with ice-cold reagents.

Brown adipose-tissue extracts for hexokinase investigations were prepared from approximately 6·5 g. of tissue by mincing with scissors and grinding at 0° in a mortar with 10 ml. glass-distilled water. The material was centrifuged for 10 min. at 2000 rev./min., the opalescent supernatant separated from the superimposed layer of fat, and employed without further treatment. The other enzyme activities were investigated in a crude extract prepared by grinding the brown adipose tissue with sand in a mortar and extracting for 10 min. with twice its weight of water. After centrifuging for 10 min. at 2000 rev./min. the supernatant was separated off. This crude tissue extract was used without further purification for the experiments on phosphoglucomutase.

Purification of phosphorylase. To the crude brown adipose-tissue extract 0·5 vol. of C_γ aluminium hydroxide suspension was added and the mixture allowed to stand at 0° for 30 min. The aluminium hydroxide was centrifuged off, washed twice with distilled water and the phosphorylase eluted for 1 hr. at room temperature with 1 vol. 0·25 M-sodium β-glycerophosphate. The mixture was centrifuged and the residue washed with 1 vol. 0·25 M-sodium β-glycerophosphate. The united eluate and washing solution was incubated at 30° for 20 min. after the addition of $\frac{1}{3}$th vol. 0·3 M-cysteine hydrochloride, pH 6·75 (Cori, Cori & Green, 1943).

Separation of phosphorylase from PR enzyme. All procedures were performed as rapidly as possible in the cold room at 5°. A crude extract of brown adipose tissue, prepared as previously described, was adjusted to pH 5·9 with N-HCl. After centrifuging off the precipitate which contained the PR enzyme, the supernatant was treated with C_γ aluminium hydroxide suspension. The phosphorylase preparation obtained from the aluminium hydroxide by elution with 0·25 M-sodium β-glycerophosphate as described above was investigated for phosphorylase activity with and without added adenylic acid.

PR enzyme. The precipitate referred to above and containing the PR enzyme was centrifuged, washed once with water and suspended in water (2 ml./g. tissue). After addition of N-KOH to pH 8·5, the mixture was frozen and allowed to thaw. The precipitate was separated by centrifugation, the supernatant adjusted to pH 6·7 and investigated for PR enzyme activity (cf. Cori & Cori, 1945).

Supplementary Enzyme was prepared by a modification of the method of Cori & Cori (1943). Saturated $(NH_4)_2SO_4$ (0·7 vol.) at pH 7·0 was added to a PR enzyme-free phosphorylase preparation (see above); the precipitated protein containing the Supplementary Enzyme was centrifuged, dissolved in distilled water (0·5 ml./g. tissue) and dialysed overnight against distilled water at 4–5°. This solution formed the Supplementary Enzyme preparation.

Determination of enzyme activity

Hexokinase was determined by the method of Colowick, Cori & Slein (1947) in which the glucose utilized on incubation of the enzyme solution with glucose and ATP at 28° is measured.

Phosphoglucomutase. Glucose-1-phosphate (1-ester) is completely hydrolysed by heating with N-H_2SO_4 at 100° for 10 min., whereas glucose-6-phosphate (6-ester) is unaffected. The conversion of 1-ester to 6-ester was, therefore, determined from the decrease in easily hydrolysable phosphate (Najjar, 1948).

Phosphorylase was determined from the percentage of inorganic P liberated by the enzyme solution from 1-ester, in the presence of adenylic acid to activate phosphorylase *b*, of NaF to inhibit phosphoglucomutase and phosphatase, and of glycogen to activate the phosphorylase (Cori *et al.* 1943).

PR enzyme. Crystalline muscle phosphorylase *a* was incubated at 25° with the PR enzyme preparation for 2 hr., samples being removed every 30 min. for determination of phosphorylase activity in the presence and in the absence of 0·3 mM-adenylic acid (Cori & Green, 1943).

Synthesis of polysaccharide by brown adipose-tissue extract. A crude extract of brown adipose tissue (1 ml.) containing 0·1 ml. 0·3 M-cysteine-glycerophosphate buffer (pH 6·75) was added to 0·5 ml. 0·28 M-1-ester (pH 6·75), 0·2 ml. 9 mM-adenylic acid, 0·1 ml. 2% glycogen and 0·2 ml. 0·2 M-NaF. After incubation for 1 hr. at 30° the reaction was stopped by the addition of 2·0 ml. 30% NaOH, and the polysaccharide determined as glycogen. In a separate experiment, after heating the reaction mixture with the NaOH, 1·3 vol. ethanol (95%) were added, the mixture boiled for a few seconds and the precipitated polysaccharide allowed to settle overnight. The mixture was then centrifuged and the polysaccharide dissolved in 1·0 ml. of water from which it was reprecipitated by the addition of 1·3 vol. of ethanol. This procedure was repeated twice, the polysaccharide washed with absolute ethanol, then with ether and finally dried *in vacuo* over anhydrous $CaCl_2$.

Supplementary Enzyme was assumed to be present in a tissue extract when the addition of that extract to a phosphorylase test system resulted in an increase in the phosphorylase activity and in the formation of a polysaccharide staining brown with iodine (Cori & Cori, 1943). The Supplementary Enzyme preparation (0·4 ml.) was added to 0·4 ml. 0·1% glycogen, 0·2 ml. 0·14 M-1-ester (pH 6·75) and 1 ml. of a solution of crystalline phosphorylase *a* in 0·03 M-cysteine-hydrochloride-glycerophosphate buffer (pH 6·75). During incubation at 30° liberation of inorganic P was determined on suitable samples as for phosphorylase activity. At the end of the reaction period the stain of the synthetic polysaccharide on addition of dilute I_2 solution was observed. Controls were also set up without added glycogen and also using boiled Supplementary Enzyme preparation. In a separate experiment using five times the above amounts the inorganic phosphate liberated indicated the conversion of 60% of the 1-ester to polysaccharide in 2·5 hr. Solid NaOH (1·5 g.) was added and the mixture heated at 100° for 30 min. The polysaccharide was then isolated as described above. The yield was 10 mg. The solution of this material in water was opalescent and stained brown with I_2. On hydrolysis with N-HCl, 4·0 mg. yielded 4·26 mg. of reducing sugar calculated as glucose.

The ability of the polysaccharide synthesized by phosphorylase *a* in the presence of the Supplementary Enzyme to prime a second phosphorylase system from which glycogen had been omitted was also investigated. The previous experiment was repeated and the incubated reaction mixture heated at 100° for 2 min. to inactivate the enzymes. After cooling, 0·4 ml. of the reaction mixture was added to 0·2 ml. 0·14 M-1-ester, 0·4 ml. water and 1 ml. of a solution of crystalline phosphorylase *a* in 0·03 M-cysteine-glycerophosphate buffer (pH 6·75) and the mixture incubated at 30°. Inorganic P liberation was determined as for phosphorylase activity. A control was set up in which the initial Supplementary Enzyme reaction mixture was heated at 100° before incubation.

RESULTS

Hexokinase

In the investigations on hexokinase activity the glucose content of the reaction mixture was not significantly changed in the absence of ATP, but in the presence of ATP two different brown adipose-tissue extracts resulted in glucose utilization of 475 and 438 μg./10 min./ml. of tissue extract. These values were of the same order as was obtained with similar extracts of muscle. The hexokinase activity of brown adipose tissue showed no definite optimum activity over the pH range 6·6–8·8. These results are in agreement with those of van Heyningen (1941) for yeast hexokinase and those of Smith (1949) for muscle hexokinase.

Phosphoglucomutase

Fig. 1 shows that in the presence of added magnesium sulphate 97·5% of the glucose-1-phosphate had been converted into glucose-6-phosphate at equilibrium. The activity was lower

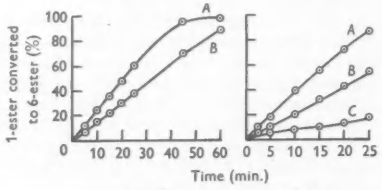

Fig. 1. Fig. 2.

Fig. 1. Phosphoglucomutase activity of 1 ml. crude extract of brown adipose tissue incubated at 30° with (*A*) 7 mM-1-ester, 2 mM-$MgSO_4$ and 25 mM-cysteine hydrochloride; pH 7·5. Total vol. = 2 ml. (*B*) $MgSO_4$ omitted.

Fig. 2. The effect of NaF on phosphoglucomutase activity of 1 ml. of a crude extract of brown adipose tissue incubated at 30° with (*A*) 7 mM-1-ester, 2 mM-$MgSO_4$ and 25 mM-cysteine hydrochloride; pH 7·5. Total vol. = 2 ml. (*B*) 1 mM-NaF added. (*C*) 3 mM-NaF added.

when the reaction mixture contained no magnesium ions other than those present in the tissue extract. Fig. 2 illustrates the inhibition of the phosphoglucomutase activity of extracts of brown adipose tissue by sodium fluoride; inhibition in the presence of 3 mM-sodium fluoride is almost complete. The initial velocity of the reaction, as measured by the amount of 6-ester phosphorus formed during the first 10 min. was plotted against the 1-ester concentration (Fig. 3). The enzyme activity was reduced at concentrations of substrate higher than 10 mM with crude aqueous extracts of adipose-tissue enzyme and above 20 mM with similar extracts of rat muscle. An apparent inhibition resulting from phosphorylase

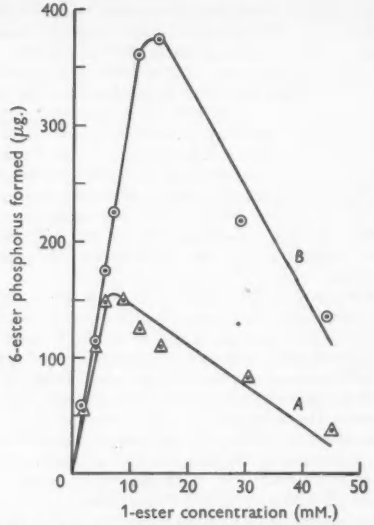

Fig. 3. Phosphoglucomutase activity expressed as μg. of 6-ester P formed in 10 min./1·0 ml. of reaction mixture when 1 ml. crude extract of brown adipose tissue (*A*) and rat muscle (*B*) was incubated at 30° with 2 mM-$MgSO_4$, 25 mM-cysteine hydrochloride and different concentrations of 1-ester; pH 7·5. Total vol. = 2 ml.

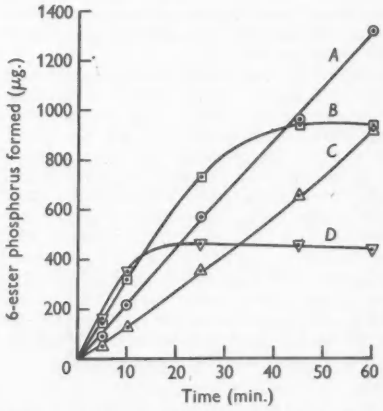

Fig. 4. Phosphoglucomutase activity of 1 ml. crude extract of brown adipose tissue incubated at 30° with 2 mM-$MgSO_4$, 25 mM-cysteine hydrochloride and different concentrations of 1-ester; pH 7·5. Total vol. = 2 ml. (*A*) With 31·6 mM-1-ester; (*B*) with 15·9 mM-1-ester; (*C*) with 47·7 mM-1-ester; (*D*) with 7·5 mM-1-ester.

or phosphatase activity becoming manifest at the higher concentrations of substrate was excluded because no inorganic phosphate was liberated. Fig. 4 shows the formation of 6-ester at different

1-ester concentrations. After 200 μg. of 6-ester phosphorus had been formed the reaction rate (as given by the slope of the curve) at a concentration of 7·55 mM-1-ester was much greater than that when the substrate concentration was 47·75 mM. The inhibition, therefore, appears due to the 1-ester itself rather than to the formation of 6-ester. The mathematical criteria of substrate inhibition suggested by Lineweaver & Burke (1934), case III, were applied to the curves in Fig. 3, but with inconclusive results.

Phosphorylase

In agreement with the observations of Cori *et al.* (1943) on muscle phosphorylase, the phosphorylase activity of purified brown adipose-tissue extracts show little or no phosphorylase activity in the absence of added cysteine. Fig. 5 shows that the phosphorylase activity of brown adipose-tissue eluate is dependent on the concentration of added glycogen. Significant amounts of inorganic phosphate were not liberated in the absence of glycogen, showing that the inorganic phosphate had not been liberated by the successive action of phosphoglucomutase and phosphatase. The phosphorylase from brown adipose tissue thus appeared to have been freed from phosphoglucomutase and phosphatase by the absorption on C_γ aluminium hydroxide. Fig. 6 shows that the phosphorylase in brown adipose tissue is inhibited by glucose, phlorrhizin and α-methyl-D-glucoside.

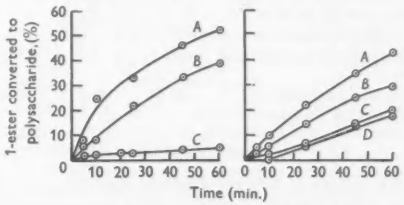

Fig. 5.

Fig. 6.

Fig. 5. Effect of glycogen concentration on the phosphorylase activity of 1 ml. brown adipose-tissue eluate containing 0·25 M-sodium β-glycerophosphate, incubated at 30° with 14 mM-1-ester, 0·3 mM-adenylic acid and 15 mM-cysteine hydrochloride; pH 6·75. Total vol. = 2 ml. (*A*) In the presence of 0·5 % glycogen; (*B*) in the presence of 0·05 % glycogen; (*C*) in the presence of 0·005 % glycogen.

Fig. 6. Phosphorylase activity of 1 ml. brown adipose-tissue eluate in the presence of inhibitors. Extract incubated at 30° with 14 mM-1-ester, 15 mM-cysteine hydrochloride, 0·3 mM-adenylic acid, 1 % glycogen; pH 6·75. Total vol. = 2 ml. (*A*) With no inhibitor; (*B*) with 0·05 M-α-methyl-D-glucoside; (*C*) with 0·05 M-glucose; (*D*) with 2·6 mM-phlorrhizin.

PR enzyme

Table 1 shows the phosphorylase activity, in the presence and in the absence of adenylic acid, of crystalline muscle phosphorylase before and after incubation at pH 6·75 with the PR enzyme preparation from brown adipose tissue. The activities are expressed as percentages of the activity, in the presence of adenylic acid, of the phosphorylase not incubated with the PR enzyme. The activity of the crystalline phosphorylase incubated in the absence of the PR enzyme remained constant during the 2 hr. period. After 30 min. incubation with the PR enzyme the activity in the absence of adenylic acid was practically abolished, but in the presence of adenylic acid the activity was still 79 % of that of the control. The phosphorylase *a* had, therefore, been converted completely to phosphorylase *b*, showing that brown adipose tissue in the rat must contain PR enzyme.

Table 1. *The activity of phosphorylase* a *after incubation with PR enzyme from brown adipose tissue*

(0·1 ml. suspension crystalline phosphorylase *a*, 0·5 ml. 0·3 M-cysteine hydrochloride (pH 6·75), 4·0 ml. PR enzyme preparation, 0·5 ml. water. Control contained water instead of PR enzyme. Incubated at 25° for 2 hr. Samples tested for phosphorylase activity with and without 0·3 mM-adenylic acid.)

	Initial activity* in the absence of PR enzyme		Activity after 30 min. incubation† with PR enzyme	
	With adenylic acid	Without adenylic acid	With adenylic acid	Without adenylic acid
1-ester converted in 5 min. (%)	57	31	50	2·1
‡$K \times 10^3$	108	43	85	2·3
Activity (%)	100	40	79	2·1

* The phosphorylase activity in the absence of PR enzyme remained unchanged during the 2 hr. incubation.
† During the next 90 min. no further change in the phosphorylase activity occurred.
‡ K is the 1st order velocity constant (Cori *et al.* 1943).

Attempts were made to remove the PR enzyme rapidly from the crude adipose-tissue extract before any phosphorylase *a*, present in the intact tissue, was converted into phosphorylase *b*. Five different extracts containing phosphorylase were prepared by the method intended to remove PR enzyme as quickly as possible. Of these extracts, four apparently contained phosphorylase *a*, i.e. they were active in the absence of added adenylic acid. Their activity was, however, increased by the addition of this substance. Fig. 7 shows the results obtained with one of these extracts, as well as those given by the fifth extract which contained phosphorylase *b*, i.e. it showed activity only in the presence of added

adenylic acid. No inorganic phosphate was liberated when glycogen was omitted from the reaction mixture so that phosphate liberation, when it occurred, must have been due to phosphorylase. It is possible that the four extracts active without added adenylic acid contained only phosphorylase *b* together with suboptimal amounts of adenylic acid derived from the original tissue. This view finds support in the fact that similar results were obtained with phosphorylase preparations in which no attempt had been made to separate PR enzyme, and in which conditions had been highly favourable for the conversion of phosphorylase *a* into phosphorylase *b*. In an attempt to free the extracts of adenylic

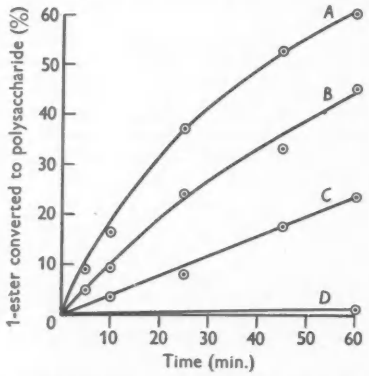

Fig. 7. Phosphorylase activity of PR-enzyme-free brown adipose-tissue extract. (*A*) and (*B*), apparent phosphorylase *a* activity of an extract; (*A*) with 0·3 mM adenylic acid; (*B*) without adenylic acid. (*C*) and (*D*), phosphorylase *b* activity of a different extract; (*C*) with 0·3 mM adenylic acid; (*D*) without adenylic acid. In each case 1 ml. of extract was incubated at 30° with 14 mM-1-ester, 15 mM-cysteine hydrochloride and 1 % glycogen; pH 6·75. Total vol. = 2 ml.

acid, several were dialysed in cellophan tubes against cysteine-glycerophosphate buffer (0·03 M, pH 6·75) at 4–5°, yet even after 8 hr. dialysis against repeatedly changed buffer solution, phosphorylase activity was still present in the absence of added adenylic acid.

Nature of the synthetic polysaccharide

Mirski (1942) showed that eluates prepared from the white adipose tissue of rats were able to catalyse the synthesis of glycogen from glucose-1-phosphate. Owing to the presence of amylase some difficulty was experienced in detecting the increase in polysaccharide concentration. Ostern, Herbert & Holmes (1939) encountered a similar difficulty in attempting to demonstrate the synthesis of polysaccharide by liver brei. By using high concentrations of glucose-1-phosphate and by adding sodium fluoride, which partially inhibited the phosphoglucomutase, Ostern *et al.* succeeded in demonstrating the synthesis of polysaccharide. Table 2 shows that under similar conditions approximately 30 % of the 1-ester was converted to polysaccharide by a crude extract of brown adipose tissue. When the polysaccharide was isolated, it behaved like glycogen in giving an opalescent solution in water and staining brown with iodine.

Table 2. *Polysaccharide synthesis by crude extracts of brown adipose tissue*

(The enzyme solution was incubated at pH 6·75 and 30° with 0·07 M-1-ester, 0·9 mM-adenylic acid, 0·1 % glycogen, 0·02 M-NaF and 0·015 M-cysteine-glycerophosphate buffer.)

	Exp. 1	Exp. 2	Exp. 3
Polysaccharide in reaction mixture at zero time (mg.)	2·9	2·5	3·4
Polysaccharide in reaction mixture after 60 min. incubation (mg.)	11·0	10·1	12·9
Polysaccharide synthesized (mg.)	8·1	7·6	9·5
Substrate converted to polysaccharide (%)	31·0	29·1	34·8

Supplementary Enzyme

Six different Supplementary Enzyme preparations from brown adipose tissue were examined.

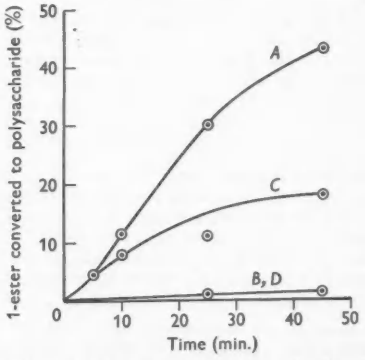

Fig. 8. Effect of brown adipose-tissue Supplementary Enzyme preparation on polysaccharide synthesis by crystalline phosphorylase *a*. Phosphorylase *a* incubated at 30° with 14 mM-1-ester, 15 mM-cysteine-glycerophosphate buffer; pH 6·75 and (*A*) Supplementary Enzyme and 0·02 % glycogen; (*B*) Supplementary Enzyme; (*C*) boiled Supplementary Enzyme and 0·02 % glycogen; (*D*) Supplementary Enzyme and 0·02 % glycogen; phosphorylase *a* omitted.

Provided a small quantity of glycogen was present to prime the reaction, all increased the activity of phosphorylase *a* and resulted in the synthesis of a

polysaccharide staining brown with iodine. Fig. 8, which is typical of the results, shows that in the absence of added glycogen there was no polysaccharide synthesis (curve *B*); after 45 min. incubation in the presence of 0·02 % glycogen, 43 % of the glucose-1-phosphate was converted into a polysaccharide staining brown with iodine (curve *A*). In the presence of boiled brown adipose-tissue extract, the reaction rate curve was typical of phosphorylase activity with low concentrations of glycogen; only 18 % of the glucose-1-phosphate was converted into polysaccharide (curve *C*) and the synthesized polysaccharide stained blue with iodine. Although 'blue' and 'A–V values' (Bourne, Haworth, Macey & Peat, 1948) were not measured, the blue colour was considered to indicate an unbranched polysaccharide molecule, whereas the brown colour was considered to indicate a highly branched polysaccharide such as glycogen. When the brown adipose-tissue Supplementary Enzyme preparation was incubated with the substrate without phosphorylase *a*, polysaccharide synthesis did not occur (curve *D*). The effect of decreasing glycogen concentration on the course of polysaccharide synthesis by the mixture of Supplementary Enzyme preparation and phosphorylase *a* is shown in Fig. 9. In the absence of glycogen no polysaccharide synthesis occurred. In the presence of 0·005 % glycogen, an autocatalytic form of curve (curve *C*) was occasionally, but not regularly, observed. Fig. 10 shows that the product of the simultaneous action of Supplementary Enzyme and crystalline phosphorylase *a* on 1-ester can prime the phosphorylase reaction. This suggests that an increase in the concentration of branched polysaccharide had occurred.

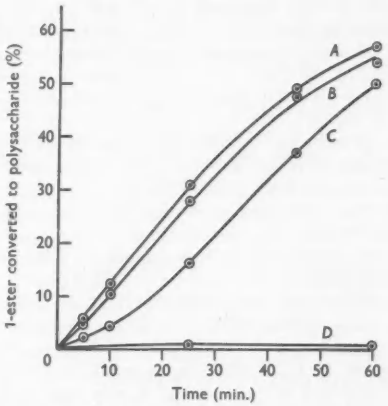

Fig. 9. Effect of brown adipose-tissue Supplementary Enzyme preparation on polysaccharide synthesis by crystalline phosphorylase *a*. Phosphorylase *a* incubated at 30° with 14 mM-1-ester, 15 mM-cysteine-glycerophosphate buffer; pH 6·75, Supplementary Enzyme and (*A*) 0·015 % glycogen; (*B*) 0·01 % glycogen; (*C*) 0·005 % glycogen; (*D*) no glycogen.

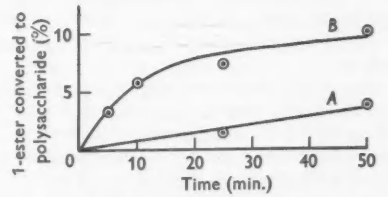

Fig. 10. Crystalline phosphorylase *a* primed with the product of simultaneous action of Supplementary Enzyme and crystalline phosphorylase *a* on 1-ester. Phosphorylase *a* incubated at 30° with 14 mM-1-ester, 15 mM-cysteine-glycerophosphate buffer (pH 6·75) and (*A*) boiled reaction mixture from experiment of Fig. 9, curve *C*, before incubation; (*B*) boiled reaction mixture from experiment of Fig. 9, curve *C*, after 1 hr. incubation.

DISCUSSION

The fact that the brown adipose tissue contains hexokinase, phosphoglucomutase, phosphorylase and Supplementary Enzyme suggests that in this tissue the synthesis of glycogen from glucose most probably occurs by a process similar to that which is known to occur in muscle. The behaviour of these enzymes is similar to that of the corresponding enzymes in muscle in regard to activators and inhibitors as far as they have been examined. Thus muscle phosphorylase is inhibited by glucose and phlorrhizin (Cori *et al.* 1943) and by α-methyl-D-glucoside (Campbell & Creasey, 1949). From the evidence presented it is impossible to decide whether brown adipose-tissue extracts possess phosphorylase *a* or phosphorylase *b* activity; although the failure to inactivate the extracts by dialysis suggests that the enzyme is in form *a*, the presence of PR enzyme in the extracts suggests that the enzyme would be in form *b*. The increase in activity of five of the preparations in the presence of adenylic acid is in agreement with the work of Cori *et al.*, who showed that phosphorylase *a* has only 71 % of its maximum activity in the absence of adenylic acid. In similar experiments, Cori & Green (1943) were unable to demonstrate phosphorylase *a* activity in heart and liver because of the high concentrations of PR enzyme in these tissues. Despite precautions to prevent the action of PR enzyme, all their extracts of heart and liver showed only phosphorylase *b* activity. The Supplementary Enzymes prepared from liver and heart by Cori & Cori (1943) showed phosphorylase *b* activity. Of the six preparations of Supplementary Enzyme from brown adipose tissue one showed no phosphorylase activity even in

the presence of adenylic acid, one showed typical phosphorylase *b* activity, while the remainder showed phosphorylase activity which was increased by the addition of adenylic acid. Similar results were obtained with seven Supplementary Enzyme preparations from liver, of which four possessed no phosphorylase activity while the other three showed activity in the absence of adenylic acid which was increased by the addition of this substance. The results are thus as equivocal as those of the experiments investigating whether phosphorylase occurs in the *a* or *b* form in brown adipose tissue.

Cori & Cori (1943) observed polysaccharide synthesis from glucose-1-phosphate by phosphorylase *a* and heart or liver Supplementary Enzyme preparations even in the absence of added glycogen, the rate curve being autocatalytic in form. In the present experiments, minute amounts of glycogen have been essential for polysaccharide synthesis (Fig. 9), and since this was also found to be the case with phosphorylase *a* and Supplementary Enzyme from liver, it may well be that Cori's preparations were not entirely free from glycogen, or other primer. Bailey, Whelan & Peat (1950) have shown that tri- and tetra-saccharides may function as primers in the synthesis of amylose by potato phosphorylase, but no information is available concerning the ability of such short chains to prime polysaccharide synthesis by animal phosphorylases, nor as to the relation of the Supplementary Enzymes of animal tissues to the corresponding *Q* enzyme of plant tissues (Barker, Bourne, Wilkinson & Peat, 1950).

Throughout the present work an alkali-stable polysaccharide staining brown with iodine has been assumed to be glycogen. It is unfortunate that we had no opportunity of measuring the optical rotation nor of the 'blue' and 'A–V' value as defined by Bourne *et al.* (1948), especially in view of the recent work of Bailey & Whelan (1950), who have shown that the *iso*phosphorylase of the potato described by Bernfeld & Meutémédian (1948) does not exist. Bernfeld & Meutémédian claimed to have prepared *iso*phosphorylase which, in the presence of potato phosphorylase and inorganic phosphorus, converted amylose into a polysaccharide presumed to possess a branched structure because it stained red with iodine. Bailey & Whelan showed that the red stain was due not to a polysaccharide with a branched structure, but to the formation of dextrins by α-amylase associated with a reduction in the amount of amylose staining blue with iodine by the action of phosphorylase in the presence of phosphate. It is clear that in future work concerning Supplementary Enzymes, care must be taken to confirm by rotation and 'A–V' measurements that the substances formed actually have branched structures.

SUMMARY

1. Hexokinase, phosphoglucomutase and phosphorylase have been demonstrated in extracts of the brown adipose tissue of the rat.

2. The phosphoglucomutase of brown adipose tissue requires magnesium ions for its optimum activity, and is inhibited by sodium fluoride and by high concentrations of glucose-1-phosphate.

3. The phosphorylase of brown adipose tissue has been prepared free from phosphatase and phosphoglucomutase; it is activated by cysteine and glycogen and inhibited by glucose, α-methyl-D-glucoside and by phlorrhizin. It was impossible to demonstrate unequivocally whether the phosphorylase in the adipose tissue was in the *a* or *b* form since the preparation obtained may have been contaminated with suboptimal concentrations of adenylic acid.

4. Extracts of brown adipose tissue contain PR enzyme.

5. The polysaccharide synthesized by the phosphorylase of brown adipose tissue stained brown with iodine and was assumed to possess a branched structure.

6. Extracts of the brown adipose tissue of the rat contained Supplementary Enzyme which, in conjunction with phosphorylase *a*, catalysed the synthesis from glucose-1-phosphate of a branched polysaccharide similar to glycogen.

We wish to express our thanks to Dr R. D. Lawrence for his criticism and advice, and also for stimulating our interest in the brown adipose tissue, to Messrs Boots Pure Drug Co. Ltd., for a personal grant to one of us (N.H.C.) and to the Central Research Fund and the Thomas Smythe Hughes Fund of the University of London for grants for special apparatus.

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Direct Oxidation of Glucose-6-phosphate, 6-Phosphogluconate and Pentose-5-phosphates by Enzymes of Animal Origin

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The existence of a direct oxidative pathway, distinct from the glycolytic route, for the oxidation of glucose-6-phosphate was demonstrated in yeast extracts by Warburg & Christian (1936, 1937), Negelein & Gerischer (1936), Lipmann (1936) and Dickens (1936, 1938*a*). Although the oxidation products were not fully identified, oxidation was considered to proceed by stepwise C-1 and C-2 oxidation and decarboxylation via 6-phosphogluconate and a pentose phosphoric ester, and Dickens (1938*a*) suggested that this was D-ribose-5-phosphate rather than D-arabinose-5-phosphate. Although the latter ester would be expected to arise from D-glucose-6-phosphate (Lipmann, 1936), of the pentose phosphates examined by Dickens (1938*a*, *b*) only D-ribose-5-phosphate was attacked by yeast enzymes at a rate justifying its assumption as an intermediate product, and the occurrence of an inversion was postulated to account for its formation.

Recent work from other laboratories, full details of which have not yet been published, tends to support this hypothesis. Cohen & McNair Scott (1950*a*), using the same yeast system as Dickens (1938*a*), identified chromatographically D-ribose-5-phosphate and possibly D-arabinose-5-phosphate among oxidation products of 6-phosphogluconate, although 50% of the pentose phosphate fraction remained unidentified. Horecker (1950) reported an enzyme preparation from yeast which converted 6-phosphogluconate quantitatively into pentose phosphate. At equilibrium (Horecker & Smyrniotis, 1950) 75% of this pentose phosphate was in the form of D-ribose-5-phosphate.

The occurrence in animal tissues of a similar oxidative pathway was indicated by preliminary experiments of Dickens (1936, 1938*a*) and the oxidation of glucose-6-phosphate by erythrocytes had already been demonstrated by Warburg & Christian (1931). Indirect physiological evidence (see Stotz, 1945) also indicated the occurrence of a non-glycolytic pathway of carbohydrate oxidation. Apart from more recent work of Barkhash & Demianovskaya (1946), Lindberg (1946) and Wainio (1947), confirming its existence, this direct oxidative pathway has not been systematically investigated in animal tissues. The present work was designed with this object in view. A preliminary account has already been published (Dickens & Glock, 1950).

MATERIALS

Hexose monophosphate (HMP). The Ba salt was prepared by the method of Ostern, Guthke & Terszakowec (1936) using starch instead of glycogen as substrate (cf. Fantl & Anderson, 1941). (Found: organic P, 7·5; inorganic P, 0·0; total hexose (Hagedorn & Jensen as modified by Robison & King, 1931), 41·6; aldose (hypoiodite method of Macleod & Robison, 1929), 29·3. Calc. for $C_6H_{11}O_5.PO_4Ba$: P, 7·8; hexose, 44·8%.)

D-*Glucose-6-phosphate* (G-6-P). The Ba salt was kindly synthesized by Dr C. T. Beer by the method of Levene & Raymond (1931). (Found: organic P, 7·5; inorganic P, 0·0; total hexose, 38·3; aldose, 39·2. Calc. for $C_6H_{11}O_5.PO_4Ba$: P, 7·8; hexose, 44·8%.)

6-*Phospho*-D-*gluconate* (6-PG) was a preparation of the Ba salt made by the late Prof. R. Robison and kindly supplied by Dr Marjorie Macfarlane. (Found: organic P, 6·7; inorganic P, 0·0; total hexose (calculated as glucose), 2·0%. Calc. for $(C_6H_{10}O_9.PO_4)_2Ba_3$: P, 6·5%.)

D-*Ribose*-5-*phosphate* (R-5-P). The Ba salt was prepared by hydrolysis of barium inosinate (Boots Pure Drug Co.) according to Levene & Jacobs (1911), and carefully freed from hypoxanthine. (Found: P, 8·9. Calc. for $C_5H_9O_4.PO_4Ba$: P, 8·5%.)

2-*Keto*-D-*gluconate*, D-*arabinose*-5-*phosphate*, *and* D-*xylose*-5-*phosphate* were preparations of the Ba salts already described (Dickens, 1938*a*, *b*).

Fructose-6-*phosphate and fructose*-1:6-*diphosphate* were kindly supplied by Dr C. S. Hanes.

Phenazine methosulphate (m.p. 160–162°) was prepared from phenazine (Light and Co.) by the method of Hillemann (1938).

Coenzyme I (*diphosphopyridine nucleotide*) (CoI) was purchased from Nutritional Biochemical Inc. Analysis (see below) showed that it contained 35·8% CoI and only 0·7% CoII.

Coenzyme II (*triphosphopyridine nucleotide*) (CoII) was prepared from horse liver by the following method kindly communicated to us by Dr C. Liébecq in 1949, and reproduced here with his permission:

'Fresh chilled horse liver (4 kg.) is finely minced through an efficient electrically driven mincer, previously cooled with ice, into cold acetone (20 l.), filtered on Büchner funnels, washed with acetone and dried in the air (yield, 1375 g. dry powder). Successive portions (300 g.) are dropped into boiling 0·5% (w/v) aqueous nicotinamide (1500 ml.), heated on a powerful gas ring so that boiling resumes within 1 min. After 2 min. further boiling, the mixture is cooled quickly, aided by the addition of 750 g. clean ice (temp. below 40° in 1 min.). The suspensions separately prepared in this way are combined, adjusted to pH 8–9 with N-NaOH and maintained at that pH while vigorously stirred for 2 hr. at room temp. The extract is separated in a press, and treated with 40 ml. 100% (w/v) trichloroacetic acid for each litre of extract. The supernatant is siphoned off and the sediment centrifuged. The combined extract is neutralized by the addition of 50% (w/v) KOH, avoiding excess.'

From the above stage Dr Liébecq's method is essentially that of Warburg, Christian & Griese (1935), being *Schritte II und III* of these authors. The final product (about 1 g. from 4 kg. liver) was still not sufficiently pure for our purpose, various batches containing from 2·8–13·8% CoI (for analytical method see below). Consequently, *Schritt III* (Warburg *et al.* 1935) was repeated on a sample which contained 25·6% CoII and 3·1% CoI, when the resulting acetone-precipitated material (0·5 g.) was almost free of CoI. (Found: 34·5% CoII, 0·6% CoI.) Unless otherwise stated, all weights of CoII given in the text refer to amounts of this specimen, and those of CoI to the sample described above. It should be noted that the precipitate of the Ba salt formed by addition of ethanol should be centrifuged immediately at room temperature, otherwise CoI is also precipitated.

Analysis of CoI *and* CoII. The reduced forms of CoI and CoII were determined spectrophotometrically at 340 mμ. in the Hilger Uvispek, using the extinction coefficients of Horecker & Kornberg (1948). CoI was determined by the method of Slater (1950) and CoII according to LePage & Mueller (1949), 'Zwischenferment' being prepared from Löwenbräu (Munich) bottom lager yeast. As an alternative method of reduction of CoII, the dehydrogenase present in liver fractions *C* (see below) obtained at 60–70% saturation with $(NH_4)_2SO_4$, together with its specific substrate, 6-phosphogluconate, proved equally suitable.

Preparation of solutions of sodium salts. The Ba salts were dissolved in the minimum of N-HCl, a slight excess of 10% (w/v) Na_2SO_4 was added, the mixture neutralized with N-NaOH, diluted to make the final substrate concentration either 0·1 or 0·05 M and centrifuged. Solutions of the Na salts were stored at −10°.

METHODS

Determination of dehydrogenase activity: (*a*) *Decolorization of brilliant cresyl blue.* A modification of Friedemann & Hollander's (1942) method was adopted, methylene blue being replaced by brilliant cresyl blue which is a more effective hydrogen acceptor in these systems (Dickens & McIlwain, 1938). To 1·0 ml. 0·1 M-(sodium) phosphate buffer (pH 7·4), 0·5 ml. 0·001 M brilliant cresyl blue, 0·1 ml. 0·05 M-substrate, 0·2 ml. (0·2 mg.) CoII and 0·7 ml. water, contained in a test tube and previously heated to 37°, were added rapidly in succession 2·5 ml. agar in phosphate buffer (Friedemann & Hollander), liquefied by warming to 42°, and 1·0 ml. enzyme solution at 37°. After mixing, the reaction mixture was solidified by placing the tubes in a freezing mixture of salt and ice and, after leaving for an additional 10 min. in a refrigerator, the decolorization times were determined at 37°. Suitable blanks (without substrate and/or coenzyme) were included in each series. The rapidity and ease of this method makes it especially useful for preliminary determinations of dehydrogenase activity in tissue extracts and also for following the CoII activity during stages in the preparation of this coenzyme.

(*b*) *Decolorization of* 2:6-*dichlorophenolindophenol.* A modification of the method of Haas (1944) was used. 0·015 M-Phosphate buffer (5 ml., pH 7·4), 0·2 ml. substrate (usually 0·1 M), 0·2 ml. CoII (or CoI) solution (usually containing 0·2 mg. coenzyme), and 1·0 ml. of a 1 in 10 dilution of the stock dye solution ($2·76 \times 10^{-3}$ M, prepared by dissolving 20 mg. 2:6-dichlorophenolindophenol in 25 ml. 0·015 M-phosphate, pH 7·4) were introduced into a 1 cm. absorptiometer cell and mixed. At t_0 min. the enzyme solution (usually 0·25 or 0·5 ml., diluted to 1·0 ml.) was added with mixing. The optical densities were read at intervals timed with a stopwatch in a Hilger Spekker absorptiometer using an Ilford spectrum red Filter no. 608. Usually two readings per minute were taken and the course was linear for at least 10 min. Blanks were run without coenzyme and substrate for each series. This method enables the initial rate of reduction to be determined, and is therefore valuable in comparative tests of activity. It is especially useful in the determination of concentration-activity relationships and for the evaluation of specificity of coenzyme and substrate.

With this method the dehydrogenase activity is expressed as μmmol. dye reduced in 1 min. (1 μmmol. or 10^{-9} g.mol./min. is equivalent to the transfer of 1·33 μl. H_2/hr.).

(*c*) *Determination of oxygen uptake.* This was determined manometrically at 37° with O_2 as the gas phase. The main part of each Warburg manometer vessel usually contained 1·0 ml. enzyme solution, 0·1 ml. 0·25 M-phosphate buffer (pH 7·0), 0·2 ml. (0·2 mg.) CoII solution, 0·2 ml. 0·05 M-substrate and 0·6 ml. water. The side bulb had 0·2 ml. aqueous phenazine methosulphate (0·3 mg.) as carrier

(Dickens & McIlwain, 1938). The centre well contained 0·2 ml. 20% (w/v) KOH as CO_2 absorbent, or in experiments with cyanide a suitable KOH-KCN mixture. After equilibration, the reaction was started by tipping in the carrier.

(*d*) *Spectrophotometric determination.* The rate of reduction of Co II was followed at 340 mμ. in 2 cm. cells in the Hilger Uvispek spectrophotometer. This method was used for determining coenzyme specificity. In the case of 6-phosphogluconate dehydrogenase, the reaction mixture consisted of 0·25 ml. (approx. 2·5 mg. dry wt.) liver fraction *C* (see below), 0·1 ml. 0·1 M-6-phosphogluconate and a solution containing 1 mg. coenzyme made up to a total volume of 4·0 ml. with 0·01 M-phosphate buffer (pH 7·4). For glucose-6-phosphate dehydrogenase activity, 0·5 ml. (approx. 4 mg. dry wt.) liver fraction *B* (see below) and 0·1 ml. 0·1 M-glucose-6-phosphate were used. The differences in densities in the presence and absence of substrates were taken as a measure of dehydrogenase activity. Suitable blanks omitting coenzyme and substrate were included.

Phosphatase activity. 1·0 ml. enzyme solution, 0·2 ml. 0·05 M-substrate and 3·8 ml. Michaelis (1931) veronal acetate buffer (pH 7·4) were incubated at 37°, and samples removed at 0, 30, 60 and 120 min. for determination of inorganic phosphate (Fiske & Subbarow, 1925) after trichloroacetic acid precipitation. Although the above conditions are presumably not optimal for phosphatase action since no Mg salts were added, they were chosen since dehydrogenase activity was measured under similar conditions. Glucose-6-phosphate, fructose-1:6-diphosphate, 6-phosphogluconate and ribose-5-phosphate were used as substrates.

Aldolase activity. The method of Herbert, Gordon, Subrahmanyan & Green (1940) was employed, except that veronal acetate buffer was used instead of borate buffer and, in addition to hexose diphosphate, ribose-5-phosphate was tested as a substrate. The liberation of free phosphate was also followed.

Phosphokinase activity. This was determined manometrically with glucose, gluconate and 2-ketogluconate as substrates by measuring the anaerobic evolution of CO_2 on addition of adenosinetriphosphate (ATP) in the presence of bicarbonate with 5% (v/v) CO_2 in N_2 as the gas phase. The main part of each Warburg manometer vessel contained 1·0 ml. enzyme solution and 1·0 ml. of a solution containing 0·02 M-$MgCl_2$ and 0·06 M-$NaHCO_3$ and the side bulb 0·1 ml. 0·1 M-substrate (Na salt) and 0·2 ml. 0·05 M-ATP (Na salt). Since the adenosinetriphosphatase (ATP-ase) activity was almost negligible, fluoride was omitted because it was not known how it might affect any phosphorylation of gluconate and 2-ketogluconate.

Preparation of tissue extracts. The fresh tissue was cooled to 0° and disintegrated thoroughly in 5 vol. ice-cold 0·01 M-phosphate buffer (pH 7·4) in the Atomix or Nelco homogenizer (supplied by Measuring and Scientific Equipment Ltd., London, S.W. 1), according to the quantity of tissue used. The homogenate was centrifuged (all centrifugation of enzymes was at 0–4°), the supernatant was siphoned off and brought to pH 4·6 at 4° with 2 N-acetic acid. After removal of the precipitate (inactive in dehydrogenating glucose-6-phosphate, 6-phosphogluconate and ribose-5-phosphate), the supernatant was brought to pH 7·0 in the cold with 2 N-NaOH, and the inactive precipitate which formed was again centrifuged off. The remaining clear solution, the volume of which was approximately that of the original homogenate, is called 'crude extract' below, and was used for rough comparison of dehydrogenase activities by the brilliant cresyl blue-agar method and as a starting material for the ammonium sulphate fractionations.

Fractional precipitation with ammonium sulphate. Sufficient solid $(NH_4)_2SO_4$ was stirred with the crude extract at pH 7 to give the desired percentage saturation at 4°. The resulting precipitates were collected by centrifugation and dialysed in cellophan tubing at 4° overnight against glass-distilled water. After dialysis the clear filtered solutions were diluted with water to one-third to one-fifth of the original volume of crude extract and stored at −10°.

If the crude extract contained glycogen this was removed by incubation with a little saliva at 37° followed by dialysis. Rabbit-liver extracts usually had much glycogen. Fasting the animals overnight was advantageous.

Occasionally, after the first addition of ammonium sulphate, the suspension was heated to 50° for 15 min. with a view to stabilizing the enzymes (Warburg & Christian, 1937), but as no increased stability was observed, heating was generally omitted.

Nucleic acid as enzyme precipitant. Fractional precipitation of the glucose-6-phosphate and 6-phosphogluconate dehydrogenases by yeast nucleic acid, in a final concentration of 0·33 mg./ml. (Kubowitz & Ott, 1942), was attempted over a pH range of 4·5–7·0.

RESULTS

Oxidation of glucose-6-phosphate and 6-phosphogluconate

The dehydrogenase activity of 'crude tissue extracts'. Dehydrogenase activity was tested by the brilliant cresyl blue method using as substrates hexose monophosphate and 6-phosphogluconate. Although the decolorization rates were found to be reasonably proportional to the amount of extract taken, the results are only semiquantitative because of some reduction of dye in absence of added substrate. This was always appreciable and was very marked with liver, presumably due to the enzymic breakdown of glycogen to glucose-6-phosphate. The blank values observed without substrate were considerably diminished when the livers of animals fasted for the previous 24 hr. were used. Dialysis was even more effective (Table 1). The reduction times (Table 1) show clearly that liver, liver carcinoma (induced by feeding *p*-dimethylaminoazobenzene to rats) and kidney, have very active Co II-coupled dehydrogenases for both hexose monophosphate and 6-phosphogluconate. Extracts of brain were also active, though less so than liver and kidney extracts. Skeletal muscle and the Rous fowl sarcoma were relatively feebly active towards both substrates. These estimations were of a preliminary nature, and the study of the distribution of the enzymes in normal and tumour tissue will be continued. The activities recorded in Table 1 are not due to adventitious blood (Warburg & Christian, 1931), since blood diluted to a haemoglobin content similar to that present in the above tissue extracts and carried through the same precipitations showed negligible dehydrogenase activity.

Table 1. *Hexose monophosphate and 6-phosphogluconate dehydrogenase activities of tissue extracts*

(Decolorization of brilliant cresyl blue with 1 ml. 'crude extract' and 0·2 mg. Co II. In the absence of Co II (or with Co I) reduction was almost negligible. The numbers in brackets show the number of samples of tissue tested.)

Tissue or organ	Substrate: None	Hexose monophosphate	6-Phosphogluconate
	Mean decolorization time (min.)		
Rat liver (9)	29	13	12
Rat liver (4)*	145	12	12
Rat liver (1)†	>600	19	5
Rabbit liver (4)	56	19	21
Horse liver (1)	105	39	46
Rat kidney (1)	117	67	62
Rabbit kidney (1)	150	41	36
Rat brain (1)	330	150	135
Rabbit brain (1)	Approx. 450	88	76
Rat skeletal muscle (2)	280	125	130
Rabbit skeletal muscle (1)	230	196	166
Rous fowl sarcoma (1)	Approx. 510	300–330	300–330
Rat-liver carcinoma (1)	65	40	11

* Animals previously fasted 24 hr.
† After dialysis.

Fractional precipitation of the dehydrogenases from 'crude extracts' of liver. Owing to its high dehydrogenase activity, liver was chosen for the attempted separation of the dehydrogenases. The following fractions were precipitated by successively adding ammonium sulphate to give the percentage saturation included in brackets:

Fractions *O* (0–40 %), *A* (40–50 %),
B (50–60 %), *C* (60–70 %).

Fractions precipitated by 70–85 and 85–100 % saturation were also collected, but the 85–100 % fraction showed no activity towards either hexose monophosphate or 6-phosphogluconate, and the 70–85 % fraction was feebly active towards the latter substrate only. Fraction *O* was also almost inactive, and fractions *A*, *B* and *C*, which contained nearly all the dehydrogenase activity, were therefore used in all subsequent experiments, either singly or as a combined 40–70 % saturation fraction, which is designated (*A* + *B* + *C*) below. The mean dry weights of fractions *A*, *B* and *C* were respectively 5·5, 6·5 and 4 % of the dry weight of liver taken.

Table 2 shows the dehydrogenase activities of the various fractions towards hexose monophosphate and 6-phosphogluconate as determined by the brilliant cresyl blue-agar or the dichlorophenolindophenol method. Co II was present in all the recorded experiments, and since the blanks with substrate but without Co II all showed negligible activity they are omitted.

The results show that the distribution of the two dehydrogenases is the same for rat and horse liver, and are summarized below:

	Fraction *A*	*B*	*C*
Hexose monophosphate dehydrogenase	+	++	–
6-Phosphogluconate dehydrogenase	+	++	+++

Table 2. *Fractional precipitation of liver dehydrogenases from 'crude extract' with ammonium sulphate*

(HMP = hexose monophosphate; 6-PG = 6-phosphogluconate.)

Species	Method*	Substrate	Saturation with $(NH_4)_2SO_4$ (%): 40–50	50–60	60–70	70–85
			Time of decolorization (min.)			
Rat	*a*	None	∞	∞	∞	∞
		HMP	66	37	∞	∞
		6-PG	270	36	10	103
Horse	*a*	None	∞	∞	∞	∞
		HMP	69	51	∞	∞
		6-PG	88	51	39	87
			mμmol. dye reduced/min.			
Rat	*b*	None	1·0	2·3	0·8	—
		HMP	10·9	14·3	1·2	—
		6-PG	7·1	16·0	18·7	—
Rabbit	*b*	None	0	1·7	15·6†	—
		HMP	0	6·9	80·4	—
		6-PG	0	4·4	82·2	—

* Method *a*. Time of decolorization of brilliant cresyl blue. Method *b*. Rate of reduction of 2:6-dichlorophenolindophenol (see Methods section).
† This high blank was due to the presence of glycogen and fell to 4·0 after digestion with saliva.

Table 3. *Substrate specificity of rat-liver fractions*

(HMP = hexose monophosphate, G-6-P = glucose-6-phosphate, 6-PG = 6-phosphogluconate, G-1-P = glucose-1-phosphate.)

Fraction	Dry wt. enzyme (mg.)	Substrate: None	HMP	G-6-P	6-PG	Glycogen	Glucose	G-1-P	Gluconate	2-Ketogluconate
		Dehydrogenase activity at 20° (μmmol./min.)*								
C, preparation 1	2·8	1·0	1·0	—	12·4	—	—	—	1·5	1·2
C, preparation 2	2·65	0	0	—	17·2	—	0	—	0·5	0·2
A+*B*+*C*, preparation 1	11·1	1·2	20·7	21·2	84·0	6·0	1·8	4·4	4·5	6·0
A+*B*+*C*, preparation 2	8·03	2·2	19·7	20·2	60·6	2·4	2·4	2·9	2·8	2·4

* μmmol. 2:6-dichlorophenolindophenol reduced/min. (see Methods section).

Fraction *C*, though richest in 6-phosphogluconate dehydrogenase, is completely free from the hexose monophosphate dehydrogenase, showing that in animal tissues, as in yeast (Dickens, 1936), these two are different enzymes. Separation of these two dehydrogenases in fraction *B* has not yet been achieved and other methods, such as electrophoresis, are being investigated. Preliminary experiments indicated that nucleic acid does not precipitate either dehydrogenase, but since many other enzymes including animal hexokinase (Glock, 1947) are precipitated, this method may prove useful for further purification of the dehydrogenases.

Although, as has already been stated, the distribution of these two dehydrogenases is similar in the rat and horse it is somewhat different in the rabbit. In the rabbit there is no clear separation of the two dehydrogenases and fraction *C* is rich in both enzymes. For this reason, rat or horse liver was used in subsequent experiments.

Substrate specificity. The specificity of the dehydrogenase preparations was tested in the presence of 0·2 mg. Co II on the combined (*A*+*B*+*C*) fraction, which has practically the whole activity, and also on fraction *C* which attacks 6-phosphogluconate but not glucose-6-phosphate. There was negligible activity in absence of substrate. Marked substrate specificity was shown by both liver fractions (Table 3).

The whole system (*A*+*B*+*C*) vigorously oxidizes Embden ester (hexose monophosphate), glucose-6-phosphate and 6-phosphogluconate. Very weak activity was shown by preparation 1 (Table 3) towards glycogen, glucose-1-phosphate, gluconate and 2-ketogluconate, but another preparation (2) had negligible activity towards these substrates. It is thus unlikely that either glucose-6-phosphate or 6-phosphogluconate dehydrogenases attacks any of these substrates. Glucose itself was not oxidized.

Fraction *C*, although highly active towards 6-phosphogluconate, did not oxidize hexose monophosphate, glucose, gluconate or 2-ketogluconate.

Coenzyme specificity. This was determined by following the reduction of Co I and Co II spectrophotometrically at 340 mμ. (see Methods section). The results (Fig. 1) show a vigorous and steady reduction of Co II by both dehydrogenase systems. With Co I there is a very brief initial reduction period which quickly (2–3 min.) ceases. The Co II content of the Co I (0·7 %) is sufficient to account for half the slight and transient activity found in the presence of Co I. The residual activity catalysed by Co I is negligible, hence both the glucose-6-phosphate and 6-phosphogluconate dehydrogenases are completely Co II specific.

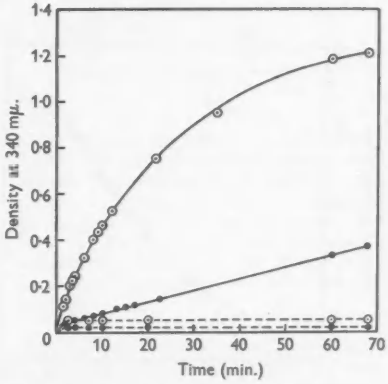

Fig. 1. Coenzyme specificity of glucose-6-phosphate and 6-phosphogluconate dehydrogenases of horse liver measured spectrophotometrically at 340 mμ. Room temperature; for details see Methods section. ⊙, 6-Phosphogluconate; ●, glucose-6-phosphate; ——, + Co II; – – –, + Co I.

Confirmation of the Co II specificity of these two systems is provided by O_2-uptake measurements. These are shown in Fig. 2, in which similar proof of the Co II specificity of ribose-5-phosphate oxidation has been included for convenience. Co I is completely ineffective in all three systems. Fig. 2 also shows that fructose-6-phosphate is oxidized in the presence of Co II almost as rapidly as glucose-6-

phosphate, whereas fructose-1:6-diphosphate is not attacked.

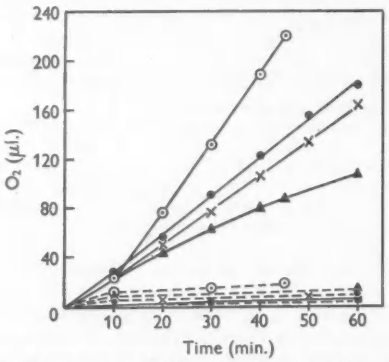

Fig. 2. Coenzyme specificity determined by O_2 uptake method. Total volume 2·5 ml. including 1 ml. horse-liver fraction $(A+B+C)$, 0·2 ml. 0·05 M-substrate, with or without the addition of 0·2 mg. Co II or Co I. 37°; for details see Methods section. ⊙, 6-Phosphogluconate; ●, glucose-6-phosphate; ▲, ribose-5-phosphate; ×, fructose-6-phosphate; ■, fructose-1:6-diphosphate; ——, +Co II; – – –, +Co I.

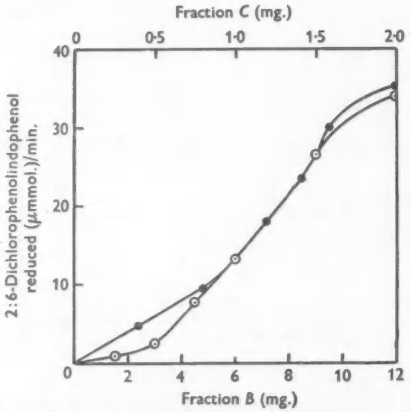

Fig. 3. Effect of enzyme concentration on the rate of dehydrogenation of glucose-6-phosphate and 6-phosphogluconate. Rate of reduction of 2:6-dichlorophenolindophenol (μmmol./min.) at 20°; total volume 7·4 ml. including 1 mg. Co II, 0·2 ml. 0·05 M-substrate and varying amounts of rat-liver fractions *B* and *C*. For details see Methods section. ●—●, Fraction *B*; substrate glucose-6-phosphate. ⊙—⊙, fraction *C*; substrate 6-phosphogluconate.

Enzyme kinetics. The 2:6-dichlorophenolindophenol reduction method was used since this permits readings of the initial rates of reaction. The concentrations of substrate, Co II and enzyme were varied, and the results are shown graphically in Figs. 3–5.

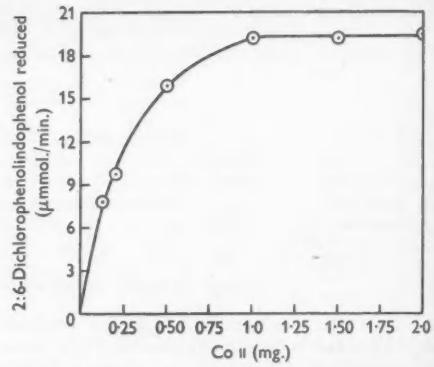

Fig. 4. Effect of Co II concentration on 6-phosphogluconate dehydrogenase activity. Rate of reduction of 2:6-dichlorophenolindophenol at 20°; 0·25 ml. rat-liver fraction $(A+B+C)$, 0·1 ml. 0·05 M-6-phosphogluconate and varying amounts of Co II (purity 34·5 %). Total vol. 7·4 ml. For details see Methods section.

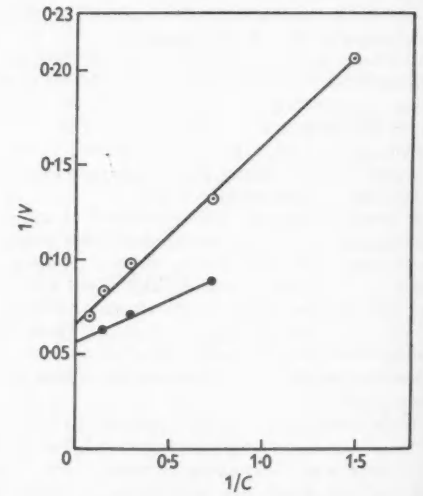

Fig. 5. Effect of substrate concentration on glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities. Rate of reduction of 2:6-dichlorophenolindophenol at 20°. Total vol. 7·4 ml. including 0·2 mg. Co II, and the following: ●—●, glucose-6-phosphate dehydrogenase (1 ml. rat-liver fraction *B*); ⊙—⊙, 6-phosphogluconate dehydrogenase (0·25 ml. rat-liver fraction *C*). V = Initial reaction velocity (μmmol. 2:6-dichlorophenolindophenol reduced/min.). C = Molar concentration of substrate. Values for $1/C$ on abscissa to be multiplied by 10^4 for glucose-6-phosphate and by 10^5 for 6-phosphogluconate dehydrogenase systems.

The enzyme concentration-activity curves (Fig. 3) are closely similar for the hexose monophosphate and 6-phosphogluconate dehydrogenases, although the activity per mg. of preparation is six times as high for the latter in the example shown. Calculated on the dry weight of enzyme taken, the Q_{H_2} values at 20° are respectively 3·4 and 22·5 μl. H_2 transferred/mg./hr., which are reasonably high considering that these preparations are certainly impure.

The effect of changes in Co II concentration on the activity of 6-phosphogluconate dehydrogenase (Fig. 4) shows that 1·0 mg. coenzyme of purity 34 % is sufficient to saturate the system contained in a total vol. of 7·4 ml., under the conditions stated.

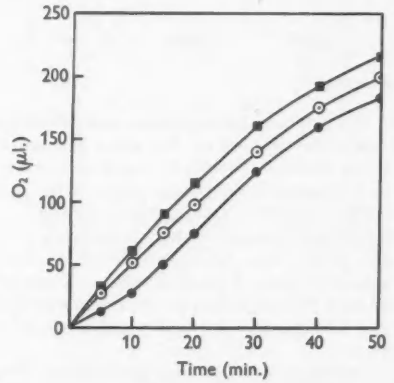

Fig. 6. Effect of cyanide on the oxidation of 6-phosphogluconate. 1 ml. rat-liver fraction *C*, 0·2 mg. Co II and 0·1 ml. 0·1 M-6-phosphogluconate. Total volume 2·5 ml.; O_2 uptake measured at 37°. ■—■, Final concentration of cyanide 0·01 M; ⊙—⊙, final concentration of cyanide 0·004 M; ●—●, no cyanide.

The effect of changes in substrate concentration on the initial reaction rates with both dehydrogenases is shown in Fig. 5. Under the conditions of these experiments 50 % of maximum initial velocity is reached with $7{\cdot}5 \times 10^{-5}$ M-hexose monophosphate and $1{\cdot}5 \times 10^{-5}$ M-6-phosphogluconate.

Time course of oxidation of glucose-6-phosphate and 6-phosphogluconate. Whereas the O_2 uptake with glucose-6-phosphate was always approximately linear, a slight lag period of about 15 min. duration was generally evident with 6-phosphogluconate oxidation (see Fig. 2). In presence of cyanide (0·01–0·004 M neutralized NaCN) this lag period is abolished, so that the initial rate was almost linear with 6-phosphogluconate and in presence of cyanide the O_2 uptake in the first 10 min. was often double that of the control without cyanide (Fig. 6). The addition of cyanide strongly promotes 6-phosphogluconate oxidation by the similar enzyme system from yeast (Dickens, 1938*a*), an effect possibly due to cyanhydrin formation with a ketonic oxidation product.

In presence or absence of cyanide, the rate of 6-phosphogluconate oxidation falls off steadily with time, and when the concentration of substrate is kept low in relation to enzyme, the end point (without cyanide) approximates to 1·5 mol. O_2/mol. phosphogluconate present (Fig. 7). This suggests that the enzyme fraction *C* is capable of oxidizing phosphogluconate to a stage beyond a pentonic acid under these conditions, the above O_2 uptake corresponding with that required for formation of a tetrose. However, the end point is not very definite and this problem can only satisfactorily be investigated by further fractionation of *C*.

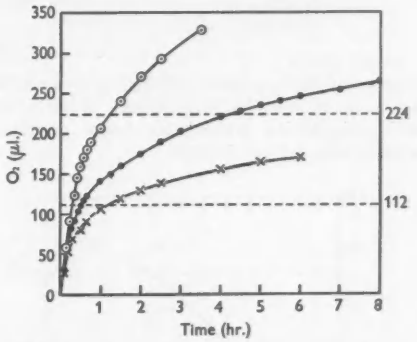

Fig. 7. Prolonged experiment on the course of oxygen uptake by the 6-phosphogluconate dehydrogenase system. Total volume 2·5 ml. including 1 ml. rat-liver fraction *C*, 1·0 mg. Co II and 6-phosphogluconate. O_2 uptake measured at 37°. ●—●, 0·1 ml. 0·1 M-substrate (calc. for 1 mol. O_2/mol. substrate = 224 μl.); ×—×, 0·05 ml. 0·1 M-substrate (calc. for 1 mol. O_2/mol. substrate = 112 μl.); ⊙—⊙, 0·1 ml. 0·1 M-substrate (a more active enzyme).

Since the slowing of oxidation in these experiments might have been due to accumulation of intermediates, e.g. a ketonic acid, the effect of cocarboxylase was tested. However, the addition of 1 mg. cocarboxylase, together with 0·1 mg. Mg^{++} and 0·1 mg. Mn^{++} had no effect on the course of oxidation of 6-phosphogluconate. The rate of reaction was also unaffected by the addition of Mg and Mn alone, contrary to the experience of Horecker (1950) with the yeast enzyme.

Oxidation of pentose phosphates

The experiments in this section are only preliminary and for brevity we have referred to the enzyme preparation which brings about the reduction of dyes in presence of pentose phosphate as pentose

Table 4. *Distribution of* D*-ribose-5-phosphate dehydrogenase and a comparison of its activity with that of glucose-6-phosphate and 6-phosphogluconate dehydrogenases*

(Brilliant cresyl blue reduction times at 37°; see Methods section.)

Source of enzyme	Substrate: None; Coenzyme: CoII	Substrate: HMP; Coenzyme: CoII	Substrate: 6-PG; Coenzyme: CoII	Substrate: R-5-P; Coenzyme: None	Substrate: R-5-P; Coenzyme: CoII
	Decolorization time (min.)				
'Crude extract' of					
Rat liver*	—	—	—	>240	97
Horse liver	105	39	46	>180	68
Rat skeletal muscle	240	115	105	>300	160
Rat kidney	—	—	—	>360	245
'Fraction $(A+B+C)$' from:					
Rat liver	>350	16	12	>350	46
Horse liver	∞	49	51	∞	70

* Average of seven batches.

phosphate dehydrogenase, although it is recognized that a more complex mechanism of reduction, possibly involving preliminary fission of the 5-carbon chain, is a possibility.

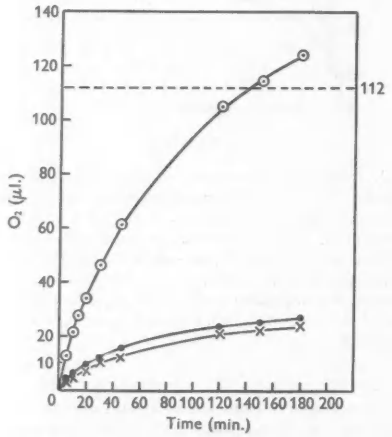

Fig. 8. Oxidation of pentose phosphates. Total volume 2·5 ml. including 1·5 ml. rat-liver fraction $(A+B+C)$, 0·2 mg. CoII and 0·1 ml. 0·05M-substrate. O_2 uptake measured at 37°. ⊙—⊙, D-Ribose-5-phosphate; ●—●, D-arabinose-5-phosphate; ×—×, D-xylose-5-phosphate. (Calc. for 1 mol. O_2/mol. substrate = 112 μl.)

Distribution of ribose-5-phosphate dehydrogenase in animal tissues. Table 4 shows the results of a number of assays by brilliant cresyl blue reduction, made either on 'crude extract' or the $(A+B+C)$ fraction. Liver again appears to be the richest source of enzyme. In all cases the dehydrogenating activity towards ribose-5-phosphate is somewhat less than that for glucose-6-phosphate and 6-phosphogluconate, determined on the same preparations. This liver fraction $(A+B+C)$ contains most of the ribose-5-phosphate dehydrogenating activity and gives a low reduction rate in absence of CoII.

The ribose-5-phosphate dehydrogenating system is less stable than glucose-6-phosphate and 6-phosphogluconate dehydrogenases. Even when stored at −10°, the ability of the enzyme to oxidize ribose-5-phosphate falls steadily and is lost in a few weeks.

Specificity towards pentose phosphates. Among the pentose-5-phosphates tested, only D-ribose-5-phosphate was oxidized rapidly, the oxidation of D-arabinose-5-phosphate and D-xylose-5-phosphate being comparatively slight (Fig. 8). This agrees with the behaviour of yeast enzymes (Dickens, 1938*a*, *b*). Unphosphorylated D-ribose was not appreciably attacked in the presence of CoII by liver fractions $(A+B+C)$ or C.

Coenzyme specificity of ribose-5-phosphate oxidation. This was determined both spectrophotometrically and by measuring the O_2 uptake. The results of the O_2 uptake measurement are shown in Fig. 2 and indicate that oxidation of ribose-5-phosphate is completely CoII specific.

Fig. 9 shows the results of the spectrophotometric measurement of reduction of CoII at 20 and 37°. For the 37° results, the reaction mixture was incubated at 37° for 15, 30 and 50 min. and then rapidly cooled to room temperature (20°) for the readings. It will be seen that the reduction of CoII is very slow at 20°, only approx. 0·2 mg. being reduced after 17 hr. This is in marked contrast to the rapid reduction of CoII by the glucose-6-phosphate and 6-phosphogluconate systems even at room temperature (Fig. 1). At 37°, however, the rate of reduction of CoII by the ribose-5-phosphate de-

hydrogenase system is rapid. Co I was not reduced by this system.

Time course of ribose-5-phosphate oxidation. Although no true end point was reached, more than 1 mol. O_2/mol. substrate was consumed (see Fig. 8).

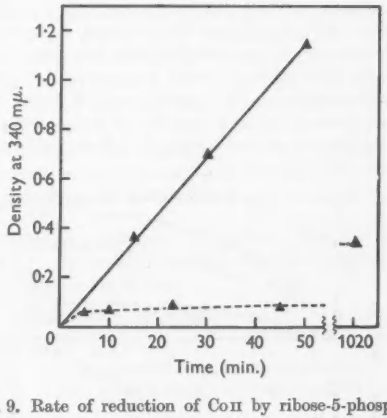

Fig. 9. Rate of reduction of Co II by ribose-5-phosphate dehydrogenase system measured spectrophotometrically at 340 mμ. Total volume 4·0 ml. including 1 ml. horse-liver fraction ($A+B+C$), 1 mg. Co II and 0·2 ml. 0·05 M-ribose-5-phosphate. Optical density at 340 mμ. measured at room temperature (20°) after incubation for different times at either 20° (▲ - - - ▲) or 37° (▲—▲).

Distribution of ribose-5-phosphate oxidizing activity in liver fractions. The activity was determined by measurement of the O_2 uptake with horse-liver fractions *O*, *A*, *B* and *C* (see p. 84) and also with combinations of these fractions, after it was found that more than one fraction was necessary for pentose phosphate oxidation (Table 5). This is clearly shown by the fact that with preparations I and II no O_2 was taken up by fractions *O*, *A*, *B* or *C* separately, but a fairly vigorous oxidation occurred in the presence of combined extracts *A*, *B* and *C*. By repeating this experiment with the combinations ($A+B$), ($A+C$) and ($B+C$), it was found that ($B+C$) together had most of the activity of ($A+B+C$).

The above experiments distinguish this dehydrogenase from those for 6-phosphogluconate (oxidized by fractions *B* and *C* separately) and glucose-6-phosphate (oxidized by fraction *B*).

In contrast to these results it was found that with another horse-liver preparation (III) fractions *A* and *C* alone were inactive, all the activity residing in fraction *B*. Since the results with preparations I and II indicated that more than one factor is necessary for the oxidation of ribose-5-phosphate, fraction *B* of Exp. III was reprecipitated with $(NH_4)_2SO_4$. The precipitates separating between 50–55, 55–60 and 60–65% saturation (called, respectively, *B*1, *B*2 and *B*3) were collected separately and their activities tested. Table 5 shows that *B*1, *B*2 and *B*3 were all inactive when tested alone. All combinations except ($B2+B3$), however, were active, most of the activity being found in ($B1+B3$). This indicates that in this experiment *B*1 is essential for ribose-5-phosphate oxidation and, in addition, another factor present in both *B*2 and *B*3 but chiefly in *B*3. Fraction *B*1 of Exp. III thus corresponds with fraction *B* of Exps. I and II and ($B2+B3$) of Exp. III with *C* of Exps. I and II. There is evidently a narrow range of $(NH_4)_2SO_4$ concentration required to give adequate separation of these fractions.

Table 5. *Enzyme fractions necessary for the oxidation of* D-*ribose-5-phosphate*

(O_2 uptake measurements at 37°. Total volume 2·5 ml. including 0·5 ml. horse-liver fractions (see text, p. 84), 0·2 mg. Co II and 0·1 ml. 0·1 M-ribose-5-phosphate.)

Preparation no.	Liver fraction	O_2 uptake (μl./60 min.)
I	*O*	0
	A	0
	B	0
	C	0
	($A+B+C$)	55
II	($A+B$)	29
	($A+C$)	55
	($B+C$)	73
	($A+B+C$)	87
III	*A*	0
	B	103
	C	0
	*B*1	0
	*B*2	0
	*B*3	0
	($B1+B2$)	50
	($B1+B3$)	84
	($B2+B3$)	0
	($B1+B2+B3$)	90

Further studies on the liver fractions

Aldolase activity. The aldolase activity of horse-liver fractions was tested towards both fructose-1:6-diphosphate and ribose-5-phosphate, using the liberation of alkali-labile phosphate as a measure of activity. It was found necessary to prolong the preliminary incubation period beyond the 4 min. used by Herbert *et al.* (1940) in order to obtain measurable rates of activity since, in accordance with Sibley & Lehninger (1949), we found the aldolase activity of liver to be considerably less than that of skeletal muscle. In addition to determining the alkali-labile phosphate, the liberation of free phosphate was also followed, since this is presumably due to phosphatase activity and should be subtracted from the 'total' phosphate (alkali-labile + free phosphate) in order to obtain a true

value for alkali-labile phosphate. Fig. 10 shows the liberation of 'total' and free phosphate from fructose-1:6-diphosphate and ribose-5-phosphate by a horse-liver fraction ($A+B+C$). This liver fraction shows marked aldolase activity with fructose-1:6-diphosphate as substrate, but the results are less clear cut with ribose-5-phosphate. After a 10 min. incubation period it would appear that the aldolase activity towards ribose-5-phosphate is approximately the same as towards fructose-1:6-diphosphate, but after this the liberation of phosphate from ribose-5-phosphate can be accounted for entirely by liberation of free phosphate.

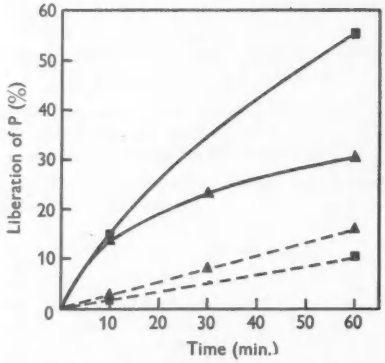

Fig. 10. Aldolase and phosphatase activity of horse-liver fraction ($A+B+C$). 2 ml. horse-liver fraction ($A+B+C$), 1 ml. veronal-acetate buffer (pH 7·4), 1 ml. 0·25 M neutralized sodium cyanide and 1 ml. 0·05 M-substrate incubated at 37° and alkali-labile and free phosphate determined at intervals. ■, Fructose-1:6-diphosphate; ▲, ribose-5-phosphate; ——, total phosphate (alkali-labile + free); – – –, free phosphate.

It was shown in the previous section that oxidation of ribose-5-phosphate by liver is dependent on the presence of at least two factors. Since it is possible that ribose-5-phosphate is first split by aldolase into a C_3 and a C_2 fragment before any oxidation can occur, the aldolase activities of liver fractions $B1$, $B2$ and $B3$ (see Table 5) were determined. These results are shown in Fig. 11. It is obvious that $B3$ contains most of the aldolase activity with fructose-1:6-diphosphate as substrate. In the case of ribose-5-phosphate, however, most phosphate is liberated by $B1$ and almost the whole of this can be accounted for by the direct liberation of free phosphate. It would thus appear that if indeed ribose-5-phosphate is split at all by a liver aldolase, this enzyme is different from that acting on fructose-1:6-diphosphate.

To exclude the possibility that what was measured as 'free' phosphate, in the case of ribose-5-phosphate, was in fact due to the formation of an acid-labile ester (e.g. ribose-1-phosphate), 'free' phosphate was estimated both by the method of Fiske & Subbarow (1925) and at pH 4 according to Lowry & Lopez (1946). The results, however, were identical.

Phosphatase activity. Fraction ($A+B+C$) of rat liver, which contains almost all the dehydrogenase activity towards glucose-6-phosphate, 6-phosphogluconate and ribose-5-phosphate, was tested (see Methods section) and found to contain no phosphatase capable of hydrolysing any of these substrates under the conditions of the dehydrogenase estimations, i.e. at the same pH (7·4) and substrate

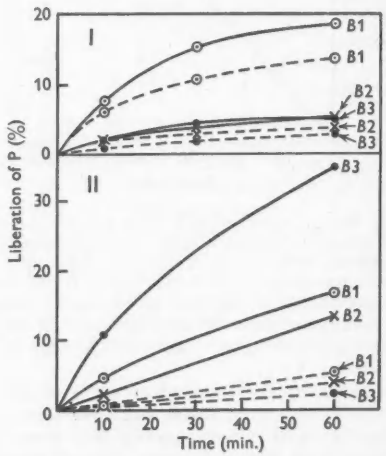

Fig. 11. Aldolase and phosphatase activity of fractions $B1$, $B2$ and $B3$. 2 ml. horse-liver fraction $B1$, $B2$ or $B3$, 1 ml. veronal-acetate buffer (pH 7·4), 1 ml. 0·25 M-cyanide and 1 ml. 0·05 M-substrate incubated at 37° and alkali-labile and free phosphate determined at intervals. I, Ribose-5-phosphate; II, fructose-1:6-diphosphate; ⊙, fraction $B1$ (50–55% saturation with $(NH_4)_2SO_4$); ×, fraction $B2$ (55–60% saturation with $(NH_4)_2SO_4$); ●, fraction $B3$ (60–65% saturation with $(NH_4)_2SO_4$); ——, alkali-labile + free phosphate; – – –, free phosphate.

concentration. No inorganic phosphate was liberated after 120 min. incubation at 37°. A similar horse-liver fraction, however, showed some activity towards both ribose-5-phosphate and fructose-1:6-diphosphate (see Fig. 10).

Since glucose-6-phosphatase has been shown to be precipitated from liver homogenates at pH 5·0 (Duve, Berthet, Hers & Dupret, 1949), this enzyme was presumably removed with the precipitate formed at pH 4·6 during the preparation of our liver extracts. Fructose-1:6-diphosphatase activity, however, is known to be stable and to remain in solution at this pH (Roche & Bouchilloux, 1950).

The effect of inorganic phosphate on the oxidation of glucose-6-phosphate, 6-phosphogluconate and ribose-5-phosphate. To study the need for inorganic phosphate the oxidative (or dehydrogenase) activity was measured at pH 7·4 (*a*) in Michaelis veronal-acetate buffer, (*b*) in phosphate buffer. The results (Table 6) do not indicate that added phosphate is essential in these reactions; veronal buffer in high concentrations inhibits the 6-phosphogluconate dehydrogenase. This lack of dependence on added phosphate is in contrast to the glycolytic breakdown of glucose-6-phosphate by yeast and animal tissues. The ethanolic fermentation of ribose-5-phosphate by yeast enzymes also requires the addition of inorganic phosphate (Dickens, 1938*b*).

Table 6. *Inorganic phosphate and the oxidation of glucose-6-phosphate, 6-phosphogluconate and ribose-5-phosphate*

(Phosphate-free liver fractions at pH 7·4 in (*a*) phosphate buffer, (*b*) veronal buffer, of concentrations as stated.)

	Rate of reduction of 2:6-dichlorophenolindophenol by 6-phosphogluconate system (fraction *C*) (μmmol./min.) at 20°				
Veronal (M)	0·01	0·02	0·02	—	—
Phosphate (M)	—	—	0·0015	0·0015	0·005
Activity	7·7	5·8	5·9	9·3	9·1

Substrate	Liver fraction	O_2 uptake (μl./60 min.) at 37°: Veronal (0·006 M)	Phosphate (0·01 M)
Glucose-6-phosphate*	(*A* + *B* + *C*)	210	202
6-Phosphogluconate*	*C*	208	195
D-Ribose-5-phosphate†	(*A* + *B* + *C*)	172	224

* Free from inorganic phosphate.

† Concentration of free phosphate initially present = 6×10^{-5} M. Calculated increase due to phosphatase action during experiment = 4×10^{-4} M-phosphate.

The effect of adenosinetriphosphate. The addition of ATP did not accelerate glucose-6-phosphate dehydrogenase activity as measured by the rate of reduction of 2:6-dichlorophenolindophenol (Table 7) or the rate of oxidation of ribose-5-phosphate by liver fraction (*A* + *B* + *C*) (Table 8). With 6-phosphogluconate there may be some inhibition of O_2 uptake (Table 9). Since the ATP-ase activity of these liver fractions was very feeble, the lack of activity of ATP is not due to its destruction by enzymic hydrolysis.

Phosphokinase activity. Rat-liver fraction (*A* + *B* + *C*) was found incapable of phosphorylating glucose, gluconate or 2-ketogluconate in the presence of ATP. Liver hexokinase was presumably inactivated or precipitated at pH 4·6 during the preparation of the extracts. No animal phosphokinase capable of phosphorylating gluconate or 2-ketogluconate has yet been described, although Cohen & McNair Scott (1950*b*) have reported the existence of a specific 'gluconokinase' in gluconate-adapted *Escherichia coli*.

Table 7. *Effect of adenosinetriphosphate on glucose-6-phosphate dehydrogenase*

(2:6-Dichlorophenolindophenol method with liver fraction (*A* + *B* + *C*); temp. 20°.)

Addition: Co II (0·2 mg.)	ATP (0·0013 M)	NaF (0·01 M)	Reduction rate (μmmol. dye/min.)
−	−	−	1·2
−	+	−	2·5
−	+	+	3·9
+	−	−	17·1
+	+	−	14·6
+	+	+	14·0

Table 8. *Effect of adenosinetriphosphate on oxidation of ribose-5-phosphate*

(Liver fraction (*A* + *B* + *C*); O_2 uptake at 37°.)

Addition: Co II	ATP	Oxygen uptake (μl./45 min.)
−	−	5
+	−	21
−	+	7
+	+	21

Table 9. *Effect of adenosinetriphosphate on oxidation of 6-phosphogluconate*

(O_2 uptake method at 37°, liver fraction *C*.)

Addition: 6-Phosphogluconate (0·01 M)	Co II (0·2 mg.)	ATP (0·01 M)	O_2 uptake (μl./120 min.)
−	+	−	3
+	−	−	1
+	+	−	121
+	−	+	7
+	+	+	91

Action of enzyme inhibitors

The activity of various enzyme inhibitors was investigated either on the dehydrogenase activities, determined by the 2:6-dichlorophenolindophenol method or on the O_2 uptakes of the dehydrogenase systems.

Cyanide and azide. Cyanide (0·01 M) produced a considerable increase (89 %) in glucose-6-phosphate dehydrogenase activity and some increase (up to 31 %) of 6-phosphogluconate dehydrogenase activity. The rate of O_2 uptake by the 6-phosphogluconate system was also increased by cyanide (0·004–0·01 M), particularly the initial stages of oxidation (Fig. 7). On the other hand, the rate of

oxidation of ribose-5-phosphate by liver fraction ($A+B+C$) was either unaffected or only slightly inhibited (< 20 %).

Azide (0·01 M) had no effect on glucose-6-phosphate or 6-phosphogluconate dehydrogenase activities, although it produced a slight increase (10–20 %) in the rate of O_2 uptake by the 6-phosphogluconate system. These facts are more in keeping with cyanohydrin formation than with inhibition of catalase as a possible explanation of the increased O_2 uptake.

Fluoride. Sodium fluoride (0·01 M) did not inhibit either glucose-6-phosphate or 6-phosphogluconate dehydrogenase activity, and the O_2 uptake of the 6-phosphogluconate and ribose-5-phosphate systems was also unaffected.

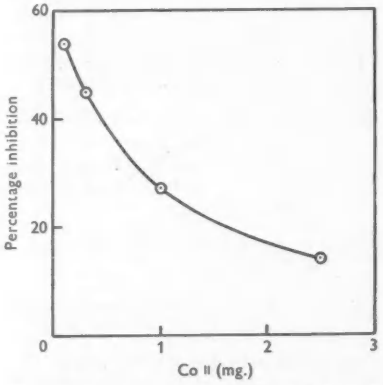

Fig. 12. Inhibition of 6-phosphogluconate dehydrogenase by nicotinamide. Rate of reduction of 2:6-dichlorophenolindophenol at 20°. Total volume 7·4 ml. including 0·5 ml. rat-liver fraction ($A+B+C$), 0·1 ml. 0·05 M-6-phosphogluconate, 40 mg. nicotinamide and varying amounts of Co II (purity 21·3 % for this experiment). Molar ratio of nicotinamide/Co II for 50 % inhibition under the above conditions = 7000/1.

Iodoacetamide. High concentrations of iodoacetamide (0·01 M) produced only a slight inhibition (< 20 %) in glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities and also of the O_2 uptakes by the 6-phosphogluconate and ribose-5-phosphate systems.

Insulin. In a few preliminary experiments, one of three samples of crystalline insulin tested (40–400 μg./ml.) was found to cause some inhibition of the glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities. Experiments are in progress to determine whether this effect can be attributed to the hyperglycaemic factor.

The effect of nicotinamide on 6-phosphogluconate dehydrogenase activity. In some preliminary experiments, estimations of enzyme activity were carried out in the presence of nicotinamide in order to diminish the enzymic breakdown of Co II. This practice was discontinued when it was observed that in the 6-phosphogluconate dehydrogenase system, nicotinamide competitively inhibited the effect of Co II (Fig. 12). Under conditions where nicotinamide produced 60 % inhibition of the above dehydrogenase system, the glucose-6-phosphate dehydrogenase system was not affected.

DISCUSSION

The present work establishes beyond doubt the existence in animal tissues of a direct oxidative pathway, distinct from the glycolytic route, for the oxidation of glucose-6-phosphate, 6-phosphogluconate and ribose-5-phosphate. In common with similar systems present in yeast, all these oxidations are Co II-specific and phenazonium salts (Dickens & McIlwain, 1938) serve as suitable carriers for aerobic oxidation. All these oxidations proceed independently of the glycolytic route since they are Co II-specific, are not inhibited by fluoride or iodoacetamide, and are not dependent on the presence of inorganic phosphate.

The mechanism of oxidative breakdown, however, requires further investigation. Since for animal tissues, as had previously been shown for yeast (Dickens, 1938*a*, *b*), D-ribose-5-phosphate is the pentose phosphate preferentially oxidized, this would support the view that D-ribose-5-phosphate is formed from D-glucose-6-phosphate by a two-stage oxidation and decarboxylation. The occurrence of D-ribose instead of the expected D-arabinose involves stereochemical inversion at the C-3 of glucose and, as was previously pointed out, 'it is recognized that inversion may occur in an asymmetrically disposed C atom with an attached H atom and an adjacent =CO group. A type of Walden inversion could theoretically occur either as a result of decarboxylation or by the introduction and subsequent removal of a second phosphoric acid group' (Dickens, 1938*a*). The occurrence of a phosphorylation appears unlikely, since in the present work we have shown that inorganic phosphate and ATP are not essential, and it is therefore more probable that inversion accompanies decarboxylation.

Fig. 13 represents a hypothetical course of oxidative formation of pentose from hexose which, being based on the intermediate formation of an enediol common to both 2- and 3-ketogluconate, appears to be consistent with the published data of Dickens (1938*a*), Cohen & McNair Scott (1950*a*, *b*), Horecker (1950), and Horecker & Smyrniotis (1950). Confirmation of this route awaits the preparation of the proposed intermediates, and it also remains uncertain whether the open-chain carboxylic acids or, as in the case of oxidation of glucose by notatin

(Bentley & Neuberger, 1949), the δ-gluconolactone (Fig. 13) is the primary oxidation product formed from glucose-6-phosphate. By analogy with free gluconic acid, 6-phosphogluconate would be expected to be in equilibrium with its lactone in neutral solution.

Moreover, less alkali-labile phosphate is split from ribose-5-phosphate by a liver fraction which splits fructose-1:6-diphosphate rapidly than by one which splits the latter substrate feebly (see Fig. 11). Thus our preliminary experiments do not favour the view that liver aldolase first splits ribose-5-phosphate

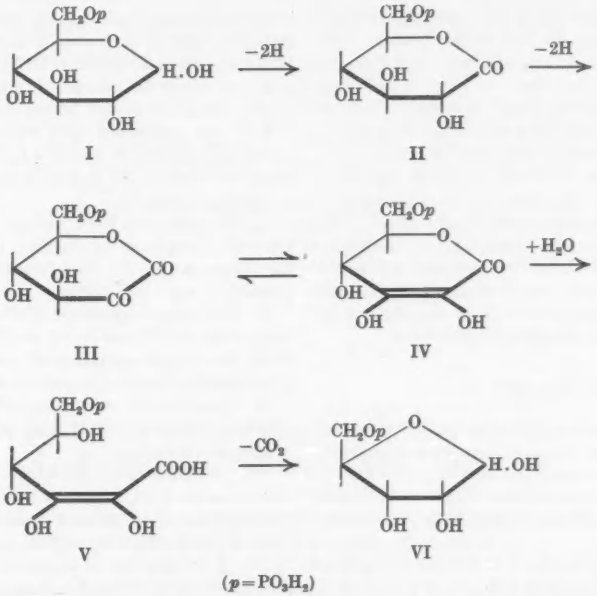

Fig. 13. Suggested route for oxidative and decarboxylative transformation of D-glucose into D-ribose. I, D-glucose-6-phosphate; II, 6-phospho-D-gluconolactone; III, 6-phospho-2-(or 3-) keto-D-gluconolactone; IV, enolic form of III; V, open-chain form of III; VI, D-ribose-5-phosphate. For the corresponding open-chain acids see Text.

The enzyme system oxidizing ribose-5-phosphate differs markedly, in some respects, from the systems oxidizing glucose-6-phosphate and 6-phosphogluconate. Thus, in the case of ribose-5-phosphate, at least two factors are necessary, which are precipitated by ammonium sulphate at different percentage saturations, both being required to effect any oxidation. In addition, the glucose-6-phosphate and 6-phosphogluconate dehydrogenase systems reduce Co II rapidly, even at room temperature, whereas with the ribose-5-phosphate dehydrogenase system, reduction of Co II is very slow at room temperature although rapid at 37°. The activity of liver aldolase has been shown by Dounce, Barnett & Beyer (1950) to decline rapidly at temperatures below 37° and extrapolation of these authors' data indicate that it would be very feeble at 20°. Although liver fractions active in oxidizing ribose-5-phosphate contain aldolase capable of splitting fructose-1:6-diphosphate, the amount of alkali-labile phosphate split from ribose-5-phosphate is very small (see Fig. 10). into a C_2 fragment and glyceraldehyde-3-phosphate before oxidation can occur, although these results remain to be substantiated by the use of other methods of determining aldolase activity.

Recent work from other laboratories, however, suggests that aldolase is involved in the metabolism of ribose-5-phosphate. Dische (1938, 1949) found that when adenosine was incubated with laked erythrocytes, pentose disappeared and was replaced by triose phosphate, hexose diphosphate and hexose monophosphate. Schlenk & Waldvogel (1946, 1947*a*) showed that under similar conditions, ribose-5-phosphate might be formed from adenosine via ribose-1-phosphate. The same authors (Schlenk & Waldvogel, 1947*b*; Waldvogel & Schlenk, 1947; Schlenk, 1949) also showed that extracts of acetone-dried rabbit liver converted ribose-5-phosphate into Robison ester. Our own experiments exclude the possibility that glucose-6-phosphate could be formed from ribose-5-phosphate via fructose-1:6-diphosphate, since this is not oxidized by liver fractions

which oxidize both ribose-5-phosphate and glucose-6-phosphate (see Fig. 2). It is possible, however, that glucose-6-phosphate could be synthesized by another route. Racker (1948, 1951) has obtained evidence of both synthesis and breakdown of a deoxypentose phosphate by an aldolase-like enzyme from *Escherichia coli*.

There seem to be two likely mechanisms of oxidation of ribose-5-phosphate by animal tissues. The first would involve splitting of ribose-5-phosphate by an aldolase, different from the enzyme splitting fructose-1:6-diphosphate, into glyceraldehyde-3-phosphate and a C_2 fragment (glycollic aldehyde?), synthesis of glucose-6-phosphate or fructose-6-phosphate from two molecules of triose and subsequent oxidation of the hexose-6-phosphate through the Co II specific systems described. The second is the oxidation of ribose-5-phosphate to 5-phospho-D-ribonic acid, with subsequent oxidative degradation as suggested by Dickens (1936, 1938*a*) for yeast. The present evidence is insufficient to decide between these alternative pathways.

SUMMARY

1. A direct oxidative pathway, distinct from the glycolytic route, for the oxidation of D-glucose-6-phosphate, 6-phospho-D-gluconate and D-ribose-5-phosphate, has been demonstrated in animal tissues and shown to resemble closely that previously described for yeast.

2. Hexose monophosphate and 6-phosphogluconate are actively oxidized by extracts of liver (horse, rat and rabbit), kidney (rat and rabbit), brain (rat and rabbit) and rat-liver carcinoma. Skeletal muscle (rat and rabbit) and Rous sarcoma of the fowl possess weak activity.

3. Ribose-5-phosphate has been shown to be oxidized by liver and kidney and less actively by skeletal muscle.

4. Oxidation of all three substrates is coenzyme II-specific.

5. These oxidations proceed independently of the glycolytic route, since inorganic phosphate is not essential for activity and high concentrations (0·01 M) of fluoride and iodoacetamide do not inhibit.

6. Partial separation of these systems from rat and horse liver has been effected by fractional ammonium sulphate precipitation. Fraction *C* (60–70 % saturation) specifically oxidizes 6-phosphogluconate. Fraction *B* (50–60 % saturation) oxidizes glucose-6-phosphate and fructose-6-phosphate but not fructose-1:6-diphosphate. Fractions *B*+*C* are generally necessary for D-ribose-5-phosphate oxidation and this is oxidized about five times as rapidly as D-arabinose-5-phosphate and D-xylose-5-phosphate.

7. At least two liver factors are necessary for ribose-5-phosphate oxidation. One of these is a coenzyme II-specific dehydrogenase and the other possibly a type of aldolase.

8. The dehydrogenation of ribose-5-phosphate is very slow at 20° but rapid at 37° and thus differs from the dehydrogenation of both glucose-6-phosphate and 6-phosphogluconate which is rapid at 20°.

9. Cyanide (0·01 M) does not inhibit the oxidation of these substrates and may accelerate the early stages of oxidation.

10. The substrate concentrations producing 50 % of the maximum initial velocities are approximately $7{\cdot}5 \times 10^{-5}$ M for hexose monophosphate and $1{\cdot}5 \times 10^{-5}$ M for 6-phosphogluconate dehydrogenases.

11. A mechanism is suggested for the oxidative formation of D-ribose-5-phosphate from D-glucose-6-phosphate.

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The Digestibility of Carotene by the Cow and the Goat as Affected by Thyroxine and Thiouracil

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It has been shown at this Institute that thyroxine affects the amounts of certain nutrients secreted in milk (Owen, 1948; Chanda & Owen, 1949; Chanda, McNaught & Owen, 1949) and that thiouracil produces opposite effects. Cama & Goodwin (1949) showed that thyroxine inhibited whilst thiouracil enhanced the loss of ingested carotene in the faeces of the rat. The present experiments were made to ascertain whether such effects were reproducible in the lactating cow and the lactating goat.

EXPERIMENTAL

(1) An experiment was carried out with six cows. It had already been shown in the goat (Chanda, Clapham, McNaught & Owen, 1951) that the Cr ratio method is as accurate as the direct method for determining the digestion of carotene. Cr_2O_3 was therefore mixed with the food fed, so as to measure the digestibility of carotene in the cows without removing them from the byre. Representative samples (1 to 2 lb.; 1 lb. = 0·45 kg.) of faeces voided between the morning milking and 11 a.m. were taken every 2nd day and analysed immediately for carotene. The cows ate only the experimental diet and were fed alternately on carotene-containing and carotene-free concentrate mixtures (compositions shown in Table 1) at the rate of 21 lb. of mixture/day in two equal portions with 10 lb. oat straw/day as roughage, irrespective of which mixture was being fed. The sole source of carotene in the rations was the dried grass meal in mixture 1 which also contained 0·1% Cr_2O_3 dispersed, as described by Chanda *et al.* (1951). Mixture 2 (designed to be free from carotene) contained blood meal, potato starch and ground straw so as to simulate the content of protein, carbohydrate and fibre in the grass meal.

Table 1. *Composition of carotene-containing and carotene-free diets for cows*

	Parts by weight	
Components	Mixture 1* (containing carotene)	Mixture 2 (carotene-free)
Oats (bruised)	9	9
Bean meal	6	6
Grass meal	6	—
Potato starch	—	2·5
Blood meal	—	0·9
Ground straw	—	2·5

* Mixture 1 contained 2100 g. Cr_2O_3 in 31·5 cwt.

There were five periods. During periods 1 and 3 the carotene-free mixture was fed, and during periods 2, 4 and 5 the mixture containing grass meal was fed. During period 4

one pair of cows received thyroxine (10 mg./cow/day) by subcutaneous injection, a second pair similarly received thiouracil (20 mg./cow/day). The third pair were controls. The treatments will be apparent from Fig. 1.

(2) Six goats were kept in metabolism crates (Chanda *et al.* 1951), specially designed so that the animals could be readily milked. The crates were provided with attachments for quantitative separation of faeces from urine. The goats were on the same diet throughout the experiment. The concentrate mixture consisted of bruised oats (9 parts by wt.), dried grass meal (6 parts) and ground nut meal (6 parts). Each goat received 3 lb./day of this mixture in three equal portions. In addition 1 lb./goat/day oat glumes in two equal portions was given as carotene-free roughage. The grass meal was the sole source of carotene. There were three periods in the experiment; 1 and 3 acted as control periods. During period 2, one pair of goats received thyroxine by subcutaneous injection (10 mg./goat/day), a second pair received thiouracil injections (20 mg./goat/day), while the remaining pair served as controls.

Methods of analysis. β-Carotene was determined in the non-saponifiable fractions of the fat of 2 g. food or 10 g. moist faeces by the method of Seshan & Sen (1942). Extracted carotenoids were purified by chromatography in light petroleum (b.p. 40–60°) on 5 × 1 cm. columns of alumina (Savory and Moore Ltd.), β-carotene being eluted by 3% acetone in light petroleum. Eluates were transferred to *n*-hexane, and made up to 50 ml., prior to determination of their extinction in a Unicam spectrophotometer. β-Carotene was calculated by assuming $E^{1\%}_{1\,cm.}$ 451 mμ. = 2500 (Morton, 1942).

After washing through with 10 ml. light petroleum the alumina columns were used again for four or five more samples. Gentle suction from a filter pump was used during chromatography, but care was taken to replenish with solvent before the tops of the columns became exposed. To keep the columns free from traces of water a little anhydrous Na_2SO_4 was placed at the top.

Cr was estimated as described by Chanda *et al.* (1951).

RESULTS

Experiment 1

The average carotene and chromium contents of the concentrate mixtures fed to the cows are shown in Table 2. There was a gradual loss of carotene in the mixture as the experiment progressed, so a fresh mixture was made at the beginning of each period using dried grass which had been stored in brown-paper bags. The average carotene contents from the beginning to the end of periods 2, 4 and 5 respectively were 5·37–4·85, 5·30–5·03 and 5·20–4·78 mg./100 g. The higher concentrations corresponded to the beginning of each period. The coefficients of variation of chromium were 15·9, 10·2 and 4·2% in periods 2, 4 and 5 respectively. The three mixtures were made in numerical order as they were needed so that Table 2 shows that efficiency of mixing increased with experience. The carotene chromium ratios used in calculating the digestibilities in Table 4 are shown in Table 2.

Table 2. *The carotene and chromium contents of carotene-containing diet for cows*

(Cr_2O_3 added for analytical purposes.)

	Period 2	Period 4	Period 5
Carotene (mg./100 g.)	5·2	5·2	5·1
Coefficient of variation*	5·1	2·2	4·6
Chromium (mg./100 g.)	69·2	69·1	71·6
Coefficient of variation*	15·9	10·2	4·2
Carotene (mg.)/chromium (g.)	75·1	74·7	71·7

* Coefficient of variation = 100 × standard deviation/mean.

The faecal excretions of carotene of three of the cows, one receiving thyroxine, one receiving thiouracil and one receiving no hormonal treatment are shown in Fig. 1. The data for the remaining three

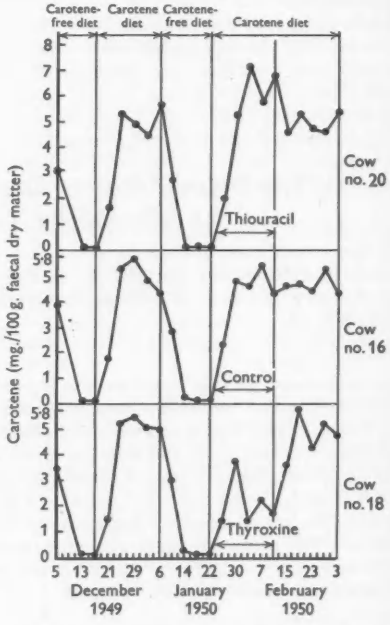

Fig. 1. Effect of thyroxine and thiouracil on the faecal excretion of carotene by cows.

cows were very similar and graphical illustrations have been omitted for brevity. Initially the concentration of carotene in the faeces of the cows varied from 2·5 to 3·8 mg./100 g. dry faeces. When the carotene-free ration was given the concentration dropped in 4 or 5 days to 100 μg./100 g. dry matter. When the carotene diet was again given the faecal carotene rose rapidly and became relatively constant in 6–8 days. The variation of faecal carotene from one cow to another (5·2–5·7 mg./100 g.) was

not very great. In period 4, during which hormonal treatments were given immediately after the carotene-free period 3, the rates of reappearance of faecal carotene were markedly affected by thyroxine. Thiouracil caused an equally marked but opposite effect (Fig. 1). These rates of reappearance have been expressed in numerical form in Table 3 which records

Table 3. *The effect of carotene-containing diet subsequent to carotene-free diet on the rate of reappearance of faecal carotene in cows*

Cow no.	Treatment in experimental period 4	Rate of increase in the faecal carotene concentration (μg./day)	
		Pretreatment period 2	Experimental period 4
15	None	266	247
16	None	286	271
17	Thyroxine	272	158
18	Thyroxine	277	188
19	Thiouracil	279	353
20	Thiouracil	284	360

the slopes (expressed as μg. increase of faecal carotene/day) of straight lines fitted by the method of least squares to the results for faecal excretion in periods 2 and 4. During period 2 the rates of reappearance of faecal carotene (Table 3) were comparable for all six cows, the average being 277 μg./day with a coefficient of variation of ±2·7%. During treatment, these rates of reappearance of faecal carotene were reduced by thyroxine to 158 μg./day with cow 17 and 188 μg./day with cow 18, and increased by thiouracil to 353 μg./day with cow 19 and 360 μg./day with cow 20.

When hormonal treatments were discontinued in period 5, the faecal carotene remained fairly constant in the controls. By contrast it increased rapidly in the cows which had received thyroxine and decreased rapidly in those which had received thiouracil (Fig. 1).

The average faecal carotene concentrations/100 g. dry faeces in periods 2, 4 and 5 of Exp. 1, when carotene was present in the diet, are shown in Table 4, together with carotene intakes and apparent digestibilities of carotene determined by the chromium sesquioxide method. The table clearly demonstrates that thyroxine increased while thiouracil diminished the digestibility of carotene by the cows.

Experiment 2

In the goat experiment, as in that with the cows, there was a progressive decrease in the concentration of carotene in the concentrate mixture which necessitated its being frequently analysed during the experiment. The concentration decreased progressively (Fig. 2), 24% of the original potency being lost in 40 days. One lot of concentrate mixture was used throughout the experiment and there were no changes of dietary regime.

The dose of thyroxine (10 mg./day) used by Meites & Turner (1948) was injected, but was too large and toxic effects supervened. One of the goats receiving

Table 4. *The effect of thyroxine and thiouracil on the faecal excretion and apparent digestibility of carotene by cows*

Cow no.	Period no.	Treatment in experimental period 4	Carotene intake (mg./2 days)	Faecal carotene (mg./100 g. dry matter)	Faecal Cr (mg./100 g. dry matter)	Carotene (mg./g. Cr in faeces)	Carotene excreted (mg./2 days)*	Apparent digestibility (%)
15	2	—	976·8	4·49	145·2	30·92	401·9	58·9
	4	None	978·3	4·25	134·6	31·58	413·8	57·7
	5	—	946·0	4·28	132·8	32·24	425·7	55·0
16	2	—	948·0	4·58	127·6	35·89	452·8	52·2
	4	None	947·4	4·29	133·6	32·11	407·4	57·0
	5	—	967·5	4·57	145·2	31·48	425·1	56·0
17	2	—	972·6	4·71	132·5	35·55	460·2	52·7
	4	Thyroxine	957·3	2·49	110·4	22·55	289·1	69·8
	5	—	957·3	4·65	153·8	30·24	404·0	57·8
18	2	—	963·4	4·79	136·3	35·14	450·5	53·2
	4	Thyroxine	980·4	2·45	122·4	20·02	262·8	73·2
	5	—	950·0	4·79	146·9	32·60	432·3	54·5
19	2	—	967·5	4·71	144·9	32·51	418·6	56·7
	4	Thiouracil	982·5	5·50	150·6	36·51	480·4	51·1
	5	—	928·5	4·85	145·9	33·24	430·8	53·6
20	2	—	928·5	4·60	137·0	33·58	414·9	55·3
	4	Thiouracil	973·2	5·35	137·2	38·98	508·0	47·8
	5	—	972·6	4·78	152·3	31·38	426·0	56·2

* Carotene intake $\times \frac{\text{mg. carotene/g. Cr in faeces}}{\text{mg. carotene/g. Cr in food (Table 2)}}$.

Table 5. *The effect of thyroxine and thiouracil on the faecal excretion and apparent digestibility of carotene by goats*

Goat no.	Period no.	Treatment in period 2	Carotene, mg./100 g. food	Carotene intake (mg./2 days)	Faecal carotene (mg./100 g. dry matter)	Faecal carotene (mg./2 days)	Apparent digestibility (%)
1	1	—	3·14	83·3	2·54	30·8	63·0
	2	None	2·97	74·1	2·35	27·4	62·3
	3	—	2·71	59·6	2·09	20·9	64·5
2	1	—	3·14	56·1	2·18	21·1	62·9
	2	None	2·97	49·0	2·05	18·9	60·7
	3	—	2·71	27·1	1·39	10·2	61·3
3	1	—	3·14	72·9	2·05	26·8	62·9
	2	Thyroxine	2·97	41·8	0·99	10·3	74·1
	3	—	2·71	65·5	2·52	27·9	57·7
4*	1	—	3·14	69·9	1·97	23·8	66·0
	2	Thyroxine	2·97	23·9	1·31	6·2	73·9
5	1	—	3·14	85·5	2·24	26·8	68·4
	2	Thiouracil	2·97	79·6	3·42	36·8	53·8
	3	—	2·71	73·7	2·49	27·9	62·1
6	1	—	3·14	82·7	2·97	32·1	61·6
	2	Thiouracil	2·97	65·5	3·37	34·8	48·0
	3	—	2·71	55·6	2·20	19·6	61·3

* This animal died from hyperthyroidism.

thyroxine died of hyperthyroidism. Nevertheless, in the goats as in the cows, the effects of thyroxine on carotene metabolism were very marked and in the opposite direction to those of thiouracil. The changes in the digestibility of carotene in the goats were of the same kind as those in the cows. The average results of the experiment are shown in Table 5, in which the digestibilities of carotene obtained by the

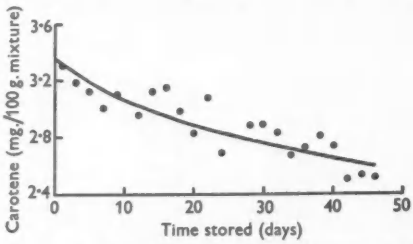

Fig. 2. Loss of carotene on storage in the concentrate mixture fed to the goats.

direct method are recorded. Table 5 also shows that thyroxine enhanced whilst thiouracil diminished the digestibility of carotene. It also shows particularly well how thyroxine reduced and thiouracil increased not only the total amount, but also the concentration of carotene in the faeces of the goats.

DISCUSSION

Cama & Goodwin (1949) found that orally administered thiouracil increased whilst thyroxine diminished the faecal carotene of rats. It would be reasonable to suppose that drugs so given would soon reach the intestinal mucosa and thus be more active in affecting an enzyme system in the intestinal wall (Glover, Goodwin & Morton, 1948; Thompson, Ganguly & Kon, 1949) than the same drugs subcutaneously injected. The present results, however, show that the effects of the drugs (1) are independent of the route of administration, (2) can be produced not only in the rat, but in both the lactating goat and the lactating cow, and (3) are not inhibited by the large fibre intake of the ruminant. If the sole site of conversion of carotene to vitamin A is the intestinal wall (Glover *et al.* 1948; Thompson *et al.* 1949) these results demonstrate hormonal control of an enzyme reaction occurring in the intestinal mucosa.

The present authors (Chanda *et al.* 1951) showed that the goat digests carotene better than does the cow. It is now found that thyroxine increases the power of the cows to digest carotene more than the power of the goats to digest carotene. On the other hand, thiouracil decreases the power of the goats to digest carotene more than the power of the cows to digest carotene. These findings are consistent with the view of Schultze & Turner (1945) that the goat thyroid gland is more active than that of the cow.

It could be argued that failure of carotene to appear in the faeces does not necessarily mean that more has been absorbed, but the observations of Seshan & Sen (1942) and of Goodwin & Gregory (1948) do not support such a criticism. Thus Goodwin & Gregory showed that carotene was not

destroyed by incubation with rabbit intestinal contents at 37°, whilst Seshan & Sen found no loss of carotene similarly incubated with cattle faeces.

The decreased concentrations of chromium in the faeces of the thyroxine-treated cows (Table 4) while intake of concentrates and roughages remained constant indicated that the digestibility of dry matter was decreased by thyroxine. A similar observation was also made earlier by Owen (1948). The digestibility of carotene was thus increased at a time when the digestibility of dry matter was reduced. This observation is consistent with the hypothesis (Glover *et al.* 1948) that carotene is converted to vitamin A in the intestinal wall.

The evidence presented here implies that the rate of conversion of carotene to vitamin A by the cow and by the goat depends on the thyroid status of the animal. Most experiments with rats indicate that 0·6 μg. β-carotene = 1 i.u. (0·3 μg.) of vitamin A (Rosenberg, 1945), but the present experiments suggest that the relationship will depend on the thyroid status of the animal.

Since the first detectable lesions of partial vitamin A deficiency in rats occur in the central nervous system (Irving & Richards, 1938), it is possible that cretinism in humans may be a result of suboptimal intake of carotene when the thyroid is damaged.

SUMMARY

The effects of thyroxine and thiouracil injections on the metabolism of carotene in the cow and the goat have been investigated with the following results.

1. Subjection of lactating cows to a diet free from carotene reduced faecal carotene to a trace in 6–8 days. Reinstatement of the carotene regime caused a rapid reappearance of faecal carotene.

2. Reappearance of faecal carotene after reinstatement of the carotene-containing diet was made markedly earlier by thiouracil and markedly later by thyroxine.

3. The digestibility of carotene by both cows and goats was markedly increased by thyroxine and markedly reduced by thiouracil.

4. Goats digested carotene more efficiently than did cows. The increase of digestibility of carotene resulting from thyroxine treatment was greater in the cows than in the goats. Conversely, the decrease due to thiouracil was greater in the goats than in the cows.

When these experiments began one of us (R.C.) was an Indian Government Scholar, but is now in receipt of a grant from the Agricultural Research Council. Grateful acknowledgement is made to both the Indian Government and the Agricultural Research Council for this financial assistance.

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The Effect of Thyroxine and Thiouracil on the Composition of Milk

1. THE PARTITION OF PHOSPHORUS IN COW MILK IN RELATION TO PHOSPHATASE AND OTHER CONSTITUENTS

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It has been shown (Owen, 1948*a*, *b*) that thyroxine increases the percentage of phosphorus in cow milk without affecting the percentages of nitrogen or calcium. Experiments were therefore planned (for preliminary reports see Chanda & Owen, 1949*a*, *b*) with the object of partitioning the phosphorus in the milk of cows treated with thyroxine to find out whether any particular type of phosphorus compound was responsible for the increase. In the earlier experiments (Owen, 1948*a*, *b*) thyroxine decreased the positivity of the balance of nitrogen. It also increased the negativity of the balance of calcium so that more calcium was lost in the faeces than was present in the food and some of the calcium in the faeces must have come from skeletal stores. Under these conditions the paradoxical observation was made that thyroxine caused the lactating cow to retain phosphorus more avidly so that metabolism of phosphorus followed neither metabolism of nitrogen nor that of calcium. The results of the present experiments help to explain this.

To throw into greater relief any chemical differences between the milk of control cows and of those receiving treatment with thryoxine, a third group of cows was treated with the antithyroid drug, thiouracil.

EXPERIMENTAL

Two experiments were made, the first with three cows and the second with five. In each experiment cows in mid-lactation and with comparable milk yields were chosen.

Lactating cows treated with thyroxine show a progressive and readily reproducible decrease in the concentration of phosphatase in the milk (Folley & White, 1936; Owen, 1948*a*). Phosphatase concentration in the milk increases with advancing lactation. In the present experiments, therefore, cows were chosen with dates of calving as near as possible to one another so as to give approximately equal initial concentrations of phosphatase in their milks.

When these experiments were envisaged we had no knowledge of the effects of injecting thiouracil into lactating animals of any species. It was known, however (Owen, 1948*a*), that 10 mg. approached the maximum safe dose of thyroxine for a lactating cow. By comparison with this injected amount, the amounts of thiouracil 100–500 mg. (British Pharmaceutical Codex, 1949) given by mouth to human patients suffering from toxic goitre appeared huge. It was therefore decided to use the phosphatase titre of the milk to compare the relative potencies of thiouracil and thyroxine. In both experiments the phosphatase was determined routinely on the milk. In Exp. 1 the effect of 10 mg. thiouracil/day was compared with the effect of 10 mg. thyroxine. In each experiment the design was the same. A period of 3 weeks, during which hormonal treatment was given, was preceded by a control period and succeeded by a recovery period. The composition of the milk during treatment could therefore be compared with the composition of the milk of the same cow before and after treatment, and simultaneously with the milk from an untreated cow. All the cows were identically managed. Milking was done by machine in the milking parlour attached to the dairy, and at every second evening milking a sample of milk (1 l.) was taken from the recorder vessel for analysis. Partitions of N and of P were determined throughout the experiments. The amounts of fat, lactose, total solids and certain other constituents in the milk were also determined and will be discussed in a subsequent communication.

Methods of analysis

P partitions were determined by a slight modification of the method of Graham & Kay (1934). P was estimated by the method of Fiske & Subbarow (1925), using a Spekker photoelectric absorptiometer. The blue colour was allowed 20 min. to develop.

Total P. An accurately weighed sample of milk was diluted with water to 10 vol. and 3 ml. of the resulting mixture were digested as described by Horecker, Ma & Haas (1940). The digest was neutralized and then diluted to 100 ml. P was determined in the resulting solution by the method of Fiske & Subbarow as described by Hawk, Oser & Summerson (1947).

Total acid-soluble P. Into a 50 ml. volumetric flask 5 ml. milk were weighed and diluted to 25 ml. with distilled water. The contents were made to the mark with 25% (w/v) trichloroacetic acid previously cooled to 4–5°. After 5 min. the contents were filtered. P was determined in 3 ml. of the filtrate after digestion as described under 'Total P'.

Inorganic phosphate. Inorganic phosphate was precipitated from 15 ml. of the filtrate from the preceding estimation contained in a 50 ml. centrifuge tube and neutralized to phenolphthalein (pH 8–9) with saturated $Ba(OH)_2$. After addition of 2 ml. 20% barium acetate the tube was left in the ice box for 20 min. prior to being centrifuged. The supernatant liquid was discarded and after the precipitate had been washed once with 10 ml. 1% barium acetate the phosphate in it was dissolved by addition of 1 drop of conc. H_2SO_4 and the contents of the tube were made to volume in a 50 ml. measuring flask. P was determined in the resulting solution as before. As a technical check inorganic P was

determined directly in the trichloroacetic acid filtrate. Table 1 demonstrates the agreement of the two methods.

Lipid P. Lipid P was extracted by ether-ethanol mixture (Hawk *et al.* 1947) and then determined, after evaporation of the ether-ethanol, exactly as for total P.

Table 1. *Ester phosphorus in milk determined either as (a) the difference between the total acid-soluble phosphorus and the barium precipitable phosphorus or as (b) the increase of phosphate by acid hydrolysis*

Sample no.	Ester P (mg. P/100 g. milk) (a)	(b)
1	7·8	7·4
2	6·2	6·0
3	8·1	7·5
4	9·2	8·7
5	9·5	9·3
6	6·1	6·4
7	8·1	7·9
8	7·3	7·5
9	7·5	7·8
10	8·3	7·9
Mean	**7·81**	**7·64**

't' (for paired difference) $= \dfrac{\text{mean difference}}{\text{S.E. of difference}} = \dfrac{0{\cdot}17}{0{\cdot}1044}$
$= 1{\cdot}628$ (not significant).

Ester P. This was calculated from the difference between the inorganic P and the total acid-soluble P.

Casein P. This was calculated from the equation

Casein P = total P − (total acid-soluble P + lipid P).

It was possible to check the reliability of the methods for ester P and casein P by other independent methods. The casein method was checked by direct precipitation of casein

Table 2. *Casein phosphorus in milk determined (a) directly in the casein precipitate or (b) from the difference between the total phosphorus and the total non-casein phosphorus*

Sample no.	Casein P (mg./100 g. milk) (a)	(b)
1	18·4	17·6
2	17·5	18·1
3	15·9	14·5
4	17·3	18·1
5	16·8	17·5
6	16·2	15·4
7	18·9	19·2
8	17·4	15·9
9	18·3	19·1
10	19·5	18·3
Mean	**17·62**	**17·37**

't' (for paired difference) $= \dfrac{0{\cdot}25}{0{\cdot}3077} = 0{\cdot}8125$ (not significant).

in the presence of an acetate buffer at pH 4 from 10 ml. milk as described by Rowland (1938), but in a 100 ml. centrifuge tube. The tubes were spun in an International centrifuge for 30 min. at 2000 rev./min. The liquid was discarded and the precipitate was washed three times with 50 ml. ether. The ether-washed casein was treated with a minimal amount of NH_3 and the resulting solution was diluted to 50 ml. with distilled water. After neutralization with N-H_2SO_4 to phenolphthalein the P content of 5 ml. of this solution was determined as already described for total P. Table 2 shows the agreement of results for casein P obtained by the two methods.

Phosphatase. Phosphatase was determined by Neave's (1939) modification of the Kay & Graham (1935) test. The short test was used in these experiments, the precautions advised by Kay, Aschaffenburg & Neave (1939) being carefully observed. Buffer substrate tablets and the Folin-Ciocalteu phenol reagent were as supplied by British Drug Houses Ltd. (B.D.H.). The amount of phenol liberated during incubation from the diphenyl phosphate present in the B.D.H. tablets was determined quantitatively in the Spekker absorptiometer. The standard graph was drawn from results obtained by using a solution containing 0·02 mg. phenol/ml. The phenol solution was standardized iodometrically (Hawk *et al.* 1947). The results for phosphatase have been recorded as mg. phenol liberated by 100 ml. milk. Such units are reproducible, but empirical, and have been called 'phenol units' in the tables and figures.

Total N. This was determined by the Kjeldahl method, 5 ml. milk being weighed and then digested with 20 ml. H_2SO_4 (sp.gr. 1·84) after addition of K_2SO_4, $CuSO_4$ and Se.

Partition of N. To obviate the necessity of washing any precipitate, the various protein fractions were determined from analysis of N in filtrates only, pipettes being calibrated for delivering solutions saturated with salt. Globulin was determined by the difference between casein, precipitated with acetate buffer as described by Rowland (1938), and casein + globulin precipitated as described by Elsdon & Walker (1942). Other protein precipitations were carried out exactly as described by Rowland (1938). The total N in each filtrate was determined by micro-Kjeldahl using the Wagner-Parnas still. When digesting the filtrate with H_2SO_4 a mixed catalyst as mentioned under 'Total N' was used except for the filtrate from the albumin + globulin precipitation, which was so rich in $MgSO_4$ that a catalyst containing only $CuSO_4$ and Se was used, the $MgSO_4$ itself serving to raise the boiling point of the H_2SO_4. NH_3 was trapped in 2% H_3BO_3 and titrated directly with 0·02 N-H_2SO_4.

RESULTS

Estimation of equivalence of responses of phosphatase to thiouracil and thyroxine

There is only speculation about the exact manner in which thiouracil inhibits thyroid activity (Andik, Balogh & Donhoffer, 1949). The toxic dose of thiouracil was not known, but it was assumed in Exp. 1 that 10 mg. thiouracil would produce a measurable response. Of the three cows compared, cow 2 received by subcutaneous injection 10 mg. thyroxine/day just after the afternoon milking, cow 3 received 10 mg. thiouracil, while cow 1 acted as an untreated control. The effects of these treatments on the phosphatase in the milk are shown in Fig. 1. The phosphatase figures for cow 2 diminished rapidly while a slight rise was shown by the

untreated animal. As was expected the phosphatase of the cow receiving thiouracil increased but at only about half the rate at which that of the thyroxine-treated cow decreased. On cessation of the treatments both effects were reversed, so that all three animals came to have comparable titres. As the rate of increase of the phosphatase titre of the milk of cow 3 was about half the rate of decrease shown by the thyroxine animal, the thiouracil dose used in Exp. 2 was doubled (20 mg.). Fig. 2 shows that the effect of this doubling was to produce rates of increase of phosphatase in the two thiouracil animals which were equal to the rates of decrease of phosphatase in the two thyroxine animals. Furthermore, as in Exp. 1, in all five animals the phosphatase titres approached the titre of the milk of the control cow as the effects of the drugs wore off. On the basis of these experiments it was assumed that, in respect of its power to change the phosphatase titre, 20 mg. thiouracil is equivalent in its effects to 10 mg. thyroxine. These doses have been used in subsequent experiments to be reported in later papers.

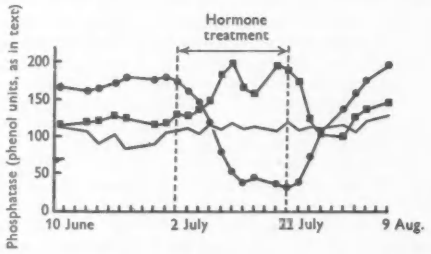

Fig. 1. Phosphatase in cow milk (Exp. 1: 1948). ■—■, cow 3 (thiouracil, 10 mg./day); ——, cow 1 (control); ●—●, cow 2 (thyroxine, 10 mg./day).

The effects of the drugs on phosphatase titre have been numerically analysed in Table 3. The data in the first column were obtained from straight lines fitted by the method of least squares to the phosphatase titres of the treatment periods shown in Figs. 1 and 2. The second column represents the intercept of the line of the first column on the phosphatase axis, i.e. it is a corrected initial titre of phosphatase. The figure for the control animals in the first column of each experiment shows the change of phosphatase which occurred in the untreated animal. The data in Table 3 amplify the conclusion drawn from Figs. 1 and 2.

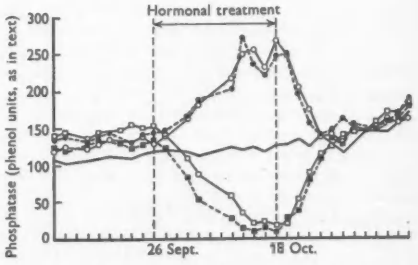

Fig. 2. Phosphatase in cow milk (Exp. 2). ○—○, cow 7 (thiouracil, 20 mg./day); ●- - -●, cow 8 (thiouracil, 20 mg./day); ——, control; □—□, cow 6 (thyroxine, 20 mg./day); ■ - - - ■, cow 5 (thyroxine 10 mg./day).

Variations of phosphatase throughout one day of treatment

Throughout these experiments the time of injection was immediately after the evening milking, and to investigate the phosphatase content of the milk throughout 1 day the five cows in Exp. 2 were milked as usual on the 15th evening, and the four experimental cows were given their usual injections.

Table 3. *Rate of change of phosphatase (phenol units) during the period of treatment*

Cow no.	Treatment	Response per day during the injection period	Intercept of regression line (theoretical initial titre)	Phosphatase on the day of maximal response*	Time required to reach maximal response (days)
		Experiment 1			
1	None	+0·37	109·3	—	—
2	Thyroxine (10 mg./day)	−7·82	162·1	39	12
3	Thiouracil (10 mg./day)	+3·25	132·9	198	10
		Experiment 2			
4	None	+0·25	117·8	—	—
5	Thyroxine (10 mg./day)	−5·84	121·7	11	18
6		−6·51	149·3	22	18
7	Thiouracil (20 mg./day)	+6·20	130·7	254	18
8		+5·01	143·1	271	16

* Minimum for thyroxine, maximum for thiouracil.

All five cows were then milked 0·5, 1, 2, 8, 12 and 16 hr. after the usual evening milking. Phosphatase in the samples was determined with the results shown in Fig. 3. Since the treatments were well advanced the phosphatase titre of the control animal was always less than that of the thiouracil-treated pair and always greater than that of the thyroxine-treated pair. There was a small initial increase in the phosphatase in the thyroxine and control animals, probably because milk collected at so short an interval after milking is very rich in fat,

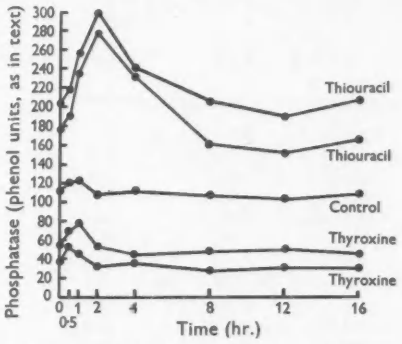

Fig. 3. Changes of phosphatase throughout the day from the time of injection. The cows which received hormonal treatment were injected at zero time on the graph.

and cream is known (Kay & Graham, 1934) to be richer in phosphatase than the remainder of the milk. A striking effect in this experiment is the large increase of phosphatase in the milk from the thiouracil animals. This shows that averages for one milking did not fully represent the effect of the thiouracil on phosphatase. This pronounced maximum in the milk phosphatase is probably attributable to ready penetration of the tissues by thiouracil and to rapid elimination of thiouracil by the kidneys (Ely, Olson & Reineke, 1948). An attempt was made to detect thiouracil in the milk by the method of Olson, Ely & Reineke (1947) but, in spite of satisfactory recoveries of thiouracil added to milk *in vitro*, none could be found. However, this was not surprising because the doses of thiouracil used (about 25 or 50 μg./kg. body weight) were minute in comparison with the dose of 100 mg./kg. body weight given subcutaneously and 200 mg./kg. given by mouth by Ely *et al.* (1948).

General effects on yields and composition

Thyroxine had its expected effect on milk yield. A graphical comparison of the effect of thyroxine and thiouracil on the milk of two of the cows is shown in Fig. 4, which also shows the percentages of fat, nitrogen and phosphorus in the milk and the percentage of the nitrogen contributed by casein. Similar data from a control cow are included. The response of the milk yield to thyroxine is typical. A noteworthy feature is the diminution of yield caused by thiouracil. There is a marked contrast between the constancy of the total phosphorus in the milk of the untreated animal and either (*a*) its increase when thyroxine is given, or (*b*) its decrease by thiouracil.

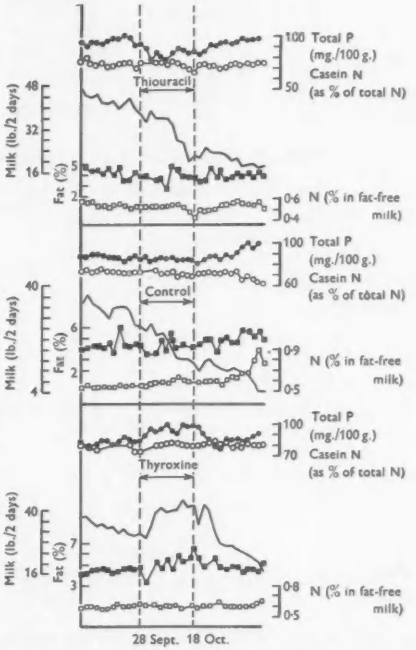

Fig. 4. Milk yield, percentages of fat, nitrogen and phosphorus and percentage of casein in total nitrogen in milk, as affected by thyroxine and thiouracil. ●—●, Total P (mg./100 g.); ○—○, casein N as % of total N; ——, milk yield; ■—■, fat (%); □—□, % N in fat-free milk.

Partition of nitrogen

Treatment with thyroxine did not affect either the total nitrogen in the milk or the percentage of casein nitrogen in the total nitrogen (Fig. 4). The effects of the drugs on the partition of nitrogen were investigated. The nitrogen partition (Table 4) shows that neither thyroxine nor thiouracil affected the partition of nitrogen between casein, albumin, globulin, proteose-peptone and non-protein nitrogen. There was no trend in the individual figures to suggest any definite effect of the drugs on the partition of nitrogen. This finding agrees with those of Van Landingham, Hyatt & Weakley (1946) and Booth, Elvehjem & Hart (1947), who partitioned the

Table 4. *Partition of nitrogen in milk from three cows given no drug, thyroxine and thiouracil respectively*

Cow no.	Period	Treatment in period 2	Total N (% by wt. of whole milk)	Percentage of total N as				
				Casein	Albumin	Globulin	Proteose-peptone	Non-protein nitrogen
1	1	—	0·604±0·045	74·6±4·5	9·0±2·2	5·4±2·0	6·8±1·6	3·6±1·0
	2	None	0·622±0·061	68·5±3·4	11·9±3·2	9·1±3·0	9·0±5·9	4·7±0·7
	3	—	0·668±0·052	64·4±5·0	3·9±0·7	14·1±3·6	13·9±3·6	4·2±1·1
2	1	—	0·592±0·057	74·0±4·1	10·1±2·8	5·8±3·9	6·4±1·2	3·8±1·0
	2	Thyroxine (10 mg./day)	0·607±0·036	71·5±2·6	12·9±3·9	5·4±3·6	9·9±4·7	4·7±0·5
	3	—	0·769±0·164	63·7±8·6	4·6±0·5	13·7±4·3	13·7±4·3	3·9±1·0
3	1	—	0·518±0·054	78·3±3·3	10·3±3·5	4·1±5·3	5·6±0·9	3·8±1·0
	2	Thiouracil (10 mg./day)	0·519±0·055	76·0±2·4	9·7±2·3	4·3±2·6	7·3±3·1	5·0±0·5
	3	—	0·537±0·051	71·0±4·5	5·4±1·4	9·3±4·1	8·4±3·1	5·8±1·2

Table 5. *Ratio of nitrogen to phosphorus in cow milk at time of maximal response**

Cow no.	Treatment in period 2	Period	Total N (mg./100 g.)	Total P (mg./100 g.)	N/P
1	—	1	599	88	6·8
	None	2	615	88	7·0
2	—	1	587	92	6·4
	Thyroxine	2	549	99	5·5
3	—	1	525	85	6·2
	Thiouracil	2	547	72	7·6
4	—	1	550	89	6·2
	None	2	564	88	6·4
5	—	1	582	86	6·8
	Thyroxine	2	577	98	5·9
6	—	1	553	90	6·1
	Thyroxine	2	519	98	5·3
7	—	1	499	91	5·5
	Thiouracil	2	496	80	6·2
8	—	1	573	99	5·8
	Thiouracil	2	612	89	6·9

* 'Maximal response' denotes the smallest value of N/P produced by thyroxine and the largest value produced by thiouracil.

nitrogen in the milk of cows receiving iodinated casein. The lack of change of the partition of nitrogen is in marked contrast to the simultaneous effects on phosphorus partition about to be described.

From Fig. 4 it can be concluded that thyroxine and thiouracil affected the ratio of total nitrogen to total phosphorus in the milk. Numerical values recorded in Table 5 show plainly that the N/P ratio which in normal circumstances appeared to average about 6·3:1 can be decreased by thyroxine to 5:1 and increased by thiouracil to 7:1.

Partition of phosphorus

The partition of phosphorus is shown in Table 6 and in Fig. 5. Table 6 shows the content of total phosphorus in the milk and its partition between inorganic phosphate, ester, lipid and casein. Averages only are recorded, and for comparison the average phosphatase titre is also shown. Comparison with Fig. 5, in which individual two-daily data for three typical cows are graphed, serves to show that the magnitudes of the hormonally induced changes of the partition of phosphorus are very inadequately represented by the averages in Table 6. The results in Fig. 5 and Table 6 confirm the earlier observation made by one of the present authors (Owen, 1948*b*) that thyroxine notably increases the percentage of phosphorus in the milk. In Figs. 4 and 5 and Tables 5 and 6 the new observation is recorded that thiouracil produces the reverse change, i.e. markedly reduces the percentage of total phosphorus in the milk. The milk of the control animals became richer in phosphorus as lactation advanced. Thyroxine enhanced while thiouracil inhibited this tendency (Table 6).

The most notable change shown in both Fig. 5 and Table 6 is that ester phosphorus was markedly increased by thyroxine and decreased by thiouracil. Lipid phosphorus changed in the same way but not so markedly. In Fig. 5; effects of thyroxine and thiouracil on casein are evident; the casein fraction from thyroxine animals became for a time richer in

phosphorus during treatment, while the casein fraction from thiouracil cows became poorer. These changes in the casein phosphorus were all the more remarkable in view of the lack of change of the casein content of the milk. Table 6 also shows that changes of ester, lipid and casein phosphorus produced by treatment with either thyroxine or thiouracil were in the opposite direction to the changes simultaneously produced in phosphatase. These inverse changes are referred to again later.

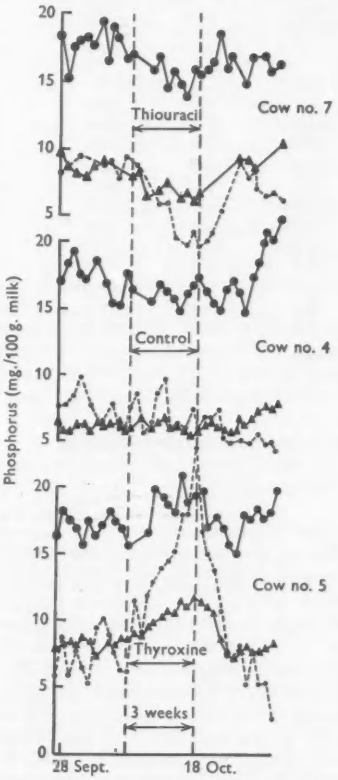

Fig. 5. Effect of thyroxine and thiouracil on the partition of phosphorus in cow milk. ●—●, Casein P; ▲—▲, lipid P; ●- - -●, ester P.

Table 6 shows that the increase of total phosphorus by thyroxine hides a decrease of inorganic phosphate, and also that the decrease of total phosphorus by thiouracil hides an increase of inorganic phosphate. Thus inorganic phosphate differs from all the other fractions investigated in changing under either treatment in the same direction as phosphatase. Comparison of Fig. 2 with Fig. 5 indicates how closely phosphatase and ester phosphorus are correlated. Accordingly coefficients of correlation of phosphatase with various phosphorus fractions have been calculated. In Tables 7 and 8 these coefficients are recorded. They show that, during either treatment, phosphatase was closely correlated negatively with ester and lipid phosphorus and positively with inorganic phosphate. Table 7 also shows that the correlation between ester

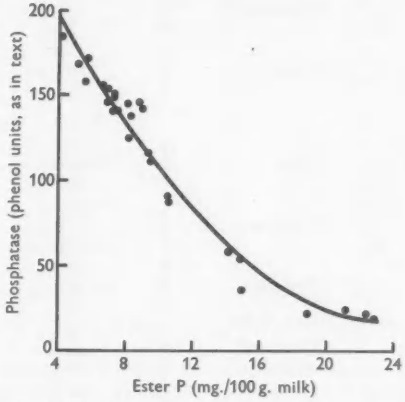

Fig. 6. Relation between ester phosphorus and phosphatase in cow no. 5 which received thyroxine.

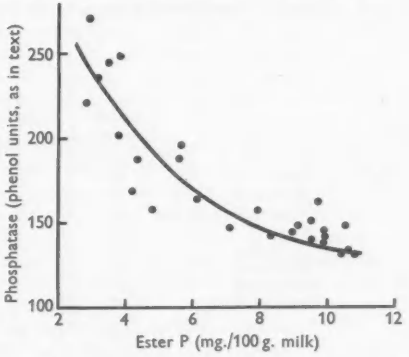

Fig. 7. Relation between ester phosphorus and phosphatase in cow no. 7 which received thiouracil.

phosphorus and phosphatase is still large during the pretreatment period and in the untreated cows. Nevertheless, the apparently small difference between the coefficients of correlation of ester phosphorus and phosphatase before and after treatment can be shown by Fisher's Z test to be significant. Differences between the ester phosphorus phosphatase correlations shown by treated and control animals were similarly shown to be

Table 6. *The effect of thyroxine and thiouracil on the partition of phosphorus*

Cow no.	Treatment in period 2	Period	Total P (mg./100 g. milk)	Percentage of total phosphorus as				Phosphatase (phenol units)
				Inorganic	Ester	Lipid	Casein	
			Experiment 1					
1	—	1	85·3	64·5	10·6	8·8	16·1	99
	None	2	86·9	63·7	10·0	8·8	17·6	113
	—	3	94·8	62·1	9·2	8·5	20·3	116
2	—	1	92·4	62·2	10·0	9·0	18·7	167
	Thyroxine	2	95·9	56·1	14·6	9·9	19·4	79
	—	3	94·5	60·9	9·3	9·0	20·7	129
3	—	1	88·2	63·4	7·6	8·6	20·4	121
	Thiouracil	2	77·7	64·8	6·5	8·3	20·4	167
	—	3	82·4	63·3	7·9	8·0	20·8	132
			Experiment 2					
4	—	1	86·7	63·9	8·9	7·2	20·1	112
	None	2	86·0	65·9	8·5	7·2	19·0	121
	—	3	90·6	66·7	6·4	7·4	19·4	129
5	—	1	82·4	56·4	10·2	11·3	22·0	129
	Thyroxine	2	92·9	51·5	17·5	12·1	20·7	43
	—	3	84·6	55·9	11·5	11·5	21·6	120
6	—	1	88·3	64·7	9·8	8·6	17·7	148
	Thyroxine	2	94·5	57·3	14·7	10·3	18·2	64
	—	3	91·8	64·2	9·6	9·4	16·8	125
7	—	1	94·4	63·1	9·5	8·8	18·9	129
	Thiouracil	2	85·1	67·8	6·1	8·1	18·2	213
	—	3	93·1	67·2	6·6	9·0	17·6	165
8	—	1	98·7	61·7	10·0	9·3	18·7	138
	Thiouracil	2	92·0	66·6	5·5	7·7	19·8	209
	—	3	98·4	66·2	7·1	9·2	17·3	170

Table 7. *Correlation of ester phosphorus and inorganic phosphorus with phosphatase*

Cow no.	Treatment in period 2	No. of observations in all periods	Correlation between ester P and phosphatase	Change of phosphatase per unit change of ester P (i.e. the linear regression coefficient)	Correlation between inorganic P expressed as % of the total P and phosphatase
1	None	30	−0·7023***	−16·2	+0·4017*
2	Thyroxine	30	−0·9469***	−17·0	+0·9658***
3	Thiouracil	30	−0·9776***	−24·3	+0·6008***
4	None	27	−0·7637***	−10·0	+0·5189**
5	Thyroxine	27	−0·9265***	−10·4	+0·8267***
6	Thyroxine	27	−0·9600***	−9·5	+0·9347***
7	Thiouracil	27	−0·9529***	−18·3	+0·8817***
8	Thiouracil	27	−0·8656***	−12·2	+0·8183***

* $P<0·05$. ** $P<0·01$. *** $P<0·001$.

significant. The same generalization does not, however, apply to the lipid phosphorus (Table 8), which in control cows and in pretreatment periods of treated cows is not significantly correlated with phosphatase at the 5% level of significance. The correlations of the ester phosphorus with phosphatase for a thyroxine and a thiouracil animal are shown in Figs. 6 and 7. Curves have been fitted to the points by the method of least squares. There is thus a curvilinear relationship between ester phosphorus and phosphatase similar to that reported for the relationship between phosphatase and ester phosphorus in human milk by Chanda, Owen & Cramond (1951).

DISCUSSION

The ratio of nitrogen to phosphorus in cow milk shows variations attributable to the thyroid gland. Thyroxine increased the phosphorus content of the milk by 15–20% without affecting the nitrogen, while thiouracil depressed the phosphorus content

Table 8. *Correlation of lipid phosphorus with phosphatase*

Cow no.	Period	Treatment in period 2	Correlation coefficient	No. of paired observations	Change of phosphatase per unit change of lipid P (i.e. the linear regression coefficient)
		Experiment 1			
1	1, 2 and 3	None	+0·2864	30	—
2	1	—	+0·3592	14	—
	2 and 3	Thyroxine	−0·9625***	16	−70
3	1	—	+0·2957	14	—
	2 and 3	Thiouracil	−0·7182**	16	−31
		Experiment 2			
4	1, 2 and 3	None	+0·0470	27	—
5	1	—	+0·1945	8	—
	2 and 3	Thyroxine	−0·9055***	19	−34
6	1	—	+0·0316	8	—
	2 and 3	Thyroxine	−0·8795***	19	−38
7	1	—	+0·0643	8	—
	2 and 3	Thiouracil	−0·7450***	19	−28
8	1	—	−0·0494	8	—
	2 and 3	Thiouracil	−0·8046***	19	−19

Unstarred figures are not significant, i.e. $P > 0\ 05$. ** $P < 0{\cdot}01$. *** $P < 0{\cdot}001$.

without any concomitant effect on nitrogen. The ratio of nitrogen to phosphorus (Table 5) was thus altered by either treatment. There is reason to believe that this ratio (N/P) is characteristic of the species. In cow milk it is only 6:1 whilst in human milk it is 20:1 (Chanda, Owen & Cramond, 1951). Even after treatment with thiouracil the N/P ratio of cow milk (7:1) is still much smaller than that of human milk.

It is known that ester phosphorus in milk acts at a suitable pH as a substrate for milk phosphatase. The present results, therefore, show that in milk the enzyme phosphatase is inversely correlated with its substrate. The part this phenomenon plays in mammary secretion is conjectural. It is noteworthy, however, that, as ester phosphorus increases, both lipid and casein phosphorus increase, partly at the expense of the inorganic phosphorus (Fig. 5 and Table 6). These observations are therefore consistent with current theories of biochemical phosphorylation, in which it is supposed that inorganic phosphate passes by way of ester phosphorus into the more complex phosphate esters found in fat and protein (Zilversmit, Entenman & Chaikoff, 1948; Popják & Muir, 1950).

When the mammary gland is made to work faster, as by thyroxine, more of the intermediate substance (ester phosphorus) is elaborated and less of the raw material (inorganic phosphate) is left. But if, as has been shown in experiments with the livers of rats by Williams-Ashman (1948), thyroxine increases the concentration of enzymes in the mammary epithelium, it is difficult to explain why, when thyroxine is given, the amount of phosphatase in the milk becomes less. Perhaps the explanation of the lessening output of phosphatase in the milk at a time when the blood contains more than usual (Folley & White, 1936) is to be correlated with the power of the cow body, when treated with thyroxine, to retain phosphorus (Owen, 1948*b*) even while losing calcium and retaining nitrogen less avidly. When the body is made to work faster by thyroxine, this avid retention of phosphorus is probably correlated with an increase in the concentration of enzymes in the body and with an increase of the energy-transferring phosphoric esters which are indispensable to their action. Under such conditions it is not surprising that the phosphorus supplies of the soft tissues are maintained partly at the expense of the bones. Phosphorus passes from the bones to the soft tissues and the equivalent of calcium passing with it from the bones is passed out of the body in the faeces of the cow (Owen, 1948*b*). That thyroxine induces large losses of calcium from the body is well known. Hunter (1930) showed this in man, Parhon & Derevici (1932) in the dog, Blaxter (1948) in the sheep and Owen (1948*b*) in the lactating cow.

It is known from work on dogs and rats (Kleiner, 1948) with phlorrhizin (which inhibits phosphorylation in the kidney) that the retention of phosphate and glucose by the kidney is due to the phosphorylation of glucose in that organ, and the classical division by Lusk (1928) of amino-acids into glucogenic and non-glucogenic was based on experiments with such phlorrhizinized dogs. It may well be that thyroxine increases the concentration of

phosphorylase in the gut thus favouring the retention of phosphorus. Such an explanation makes it necessary, however, to postulate quite different conditions in the mammary gland which secretes phosphoric esters in greater amounts at the same time as its output of phosphatase decreases. It has been shown by Houston, Kon & Thompson (1940) and by Chanda, McNaught & Owen (1949) that phosphorylated aneurin is closely correlated with phosphatase. Chanda *et al.* (1951) found a similar negative correlation between phosphorylated aneurin and phosphatase in human milk. In cows even protein-bound aneurin varies in the opposite direction to phosphatase during treatment with either thyroxine or thiouracil (Chanda *et al.* 1949). With thiouracil, conditions in the mammary gland are such that more phosphatase appears in the milk. This observation makes it unlikely that phosphatase in milk is a 'leakage' product, for with thiouracil the escape of a smaller molecule (phosphorylated aneurin) (Chanda *et al.* 1949) is inhibited, while that of a larger one (phosphatase) is facilitated. Even a molecule so small as acetic acid does not appear in the milk as such in spite of its presence (McClymont, 1949) in ruminant blood and in the glycerides of ruminant milk fat (Achaya & Hilditch, 1950; French, Hunter, Martin & Popják, 1951).

A leakage hypothesis is hardly compatible with the correlations between phosphatase and the various phosphorus fractions reported in the present paper.

The changes produced by thyroxine and thiouracil in the present experiments imply that the phosphorus content of casein is affected by thyroid activity. This may, therefore, account for the difficulty of preparing casein of constant phosphorus content (Elsdon & Walker, 1942; Associates of Rogers, 1935).

SUMMARY

1. The main results on cow milk of thyroxine and thiouracil injections were: (i) thiouracil increased the phosphatase titre; (ii) comparison of the rates of change of phosphatase after thyroxine or thiouracil treatments showed that 20 mg. thiouracil gave an increase of the same magnitude as the decrease caused by 10 mg. thyroxine; (iii) milking at frequent intervals during 24 hr. showed that 2 hr. after a normal evening milking there is a pronounced maximum in the phosphatase titre of cows treated with thiouracil; (iv) neither thyroxine nor thiouracil affected the partition of nitrogen between casein, albumin, globulin, proteose-peptone and non-protein nitrogen; (v) thyroxine increased while thiouracil decreased the content of phosphorus of the milk; (vi) thyroxine decreased while thiouracil increased the ratio of total nitrogen to total phosphorus in the milk; (vii) thyroxine increased ester phosphorus markedly and also increased lipid phosphorus and caused a transient increase in casein phosphorus, partly at the expense of inorganic phosphorus; (viii) thiouracil affected the partition of phosphorus in the milk in exactly the opposite way to thyroxine.

2. (i) As a result of changes in phosphatase and phosphorus partition there were negative correlations between phosphatase on the one hand and ester phosphorus and lipid phosphorus on the other. Inorganic phosphate was positively correlated with phosphatase; (ii) large negative coefficients of correlation between ester phosphorus and phosphatase were found in untreated cows and in the experimental cows before treatment. These coefficients became larger under the influence of either drug.

3. It is suggested in the light of this and earlier experiments that the effect of thyroxine in increasing the metabolic rate may be due to an increase of the phosphorylating enzymes in the tissues.

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Non-peptide Linkages in Gramicidin

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Gregory & Craig (1948; cf. Craig, Gregory & Barry, 1950) showed by counter-current distribution that crystalline gramicidin as usually prepared is a mixture of at least three closely related substances. That present in largest amount (*A*) contained the residues previously reported, whereas *B* contained in addition phenylalanine and *C* tyrosine. The work described in the present paper was done with samples of gramicidin obtained from tyrothricin by simple solvent extraction and crystallization, and hence contained at least these three components. Despite this complication it is felt that the information gained should cast some light on structural features that may be common to members of the group of closely related substances known as gramicidin.

We undertook this work in the hope of gaining information as to how gramicidin, apparently made up entirely from residues of monoamino-monocarboxylic acids and ethanolamine, is nevertheless devoid of basic properties and ionizing groups. A general review of previous data on the structure of gramicidin has been given by Synge (1950; cf. Hodgkin, 1950), and this includes a brief statement of the problem with a preliminary account of some of our present data. Some of the information given in that review needs modification in view of the discovery by Hinman, Caron & Christensen (1950) that the racemic material obtained on partial acid hydrolysis which was thought by Christensen (1943, 1944) to be an equimolecular mixture of the peptides D-valyl-D-valine and L-valyl-L-valine is more probably a mixture of D-valyl-L-valine and L-valyl-D-valine. While the experiments now described do not lead to any rigorous conclusions as to structure they at least rule out a number of possible solutions of the problem.

It has been established by a number of methods that the gramicidins are neutral substances possessing no free amino or carboxyl groups. No free ionizing groups were found on electrometric titration in 60% (v/v) aqueous ethanol by Christensen, Edwards & Piersma (1941; confirmed in present paper, cf. Fig. 1), on titration in ethanol and in acetone by Hotchkiss (1941) or on titration in glacial acetic acid with perchloric acid (present paper). No amino groups were detected by Hotchkiss (1941) either by reaction with nitrous acid according to Van Slyke or by the colour reaction with ninhydrin. Similarly, we could obtain no stable yellow *N*-dinitrophenyl (*N*-DNP) derivative by reaction with 1-fluoro-2:4-dinitrobenzene (FDNB), and the migration behaviour of gramicidin in an electric field at pH 6 was no different from that of the neutral substance *N*-DNP-ethanolamine under the same conditions.

The only free functional groups of gramicidin other than indole groups seem to be hydroxyl groups possessing an alcoholic function. Gramicidin

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is readily acetylated by acetic anhydride (Hotchkiss, 1941; Fraenkel-Conrat, Brandon & Olcott, 1947); it gives a succinyl derivative with succinic anhydride (Olcott, Lewis, Dimick, Fevold & Fraenkel-Conrat, 1946), a sulphuric half ester with cold concentrated sulphuric acid (Reitz, Ferrel, Fraenkel-Conrat & Olcott, 1946) and a phosphoric ester with phosphorus pentoxide in phosphoric acid (Ferrel, Olcott & Fraenkel-Conrat, 1948). It has now been shown that these hydroxyl groups can be methylated by means of methyl iodide and silver oxide. Table 1 gives a comparison of the number of gram residues of each of these groups introduced into gramicidin together with the number of gram residues of ethanolamine present in acid hydrolysates of the same amount of gramicidin. The agreement between these sets of figures suggests that the number of free hydroxyl groups is equal to the number of ethanolamine residues, and hence that the former derive from the latter. This would imply that ethanolamine residues are incorporated in the gramicidin molecule by a peptide bond on the amino group, thus: $R.CO.NH.CH_2.CH_2.OH$, where R signifies the remainder of the gramicidin molecule. Methylation of gramicidin would then be expected to give the derivative $R.CO.NH.CH_2.CH_2.OCH_3$. Hydrolysis of the methylated product with an acid that would not split the ether linkage should then give rise not to ethanolamine but to *O*-methylethanolamine, $NH_2CH_2.CH_2OCH_3$. However, this was not found; complete hydrolysis of methylated gramicidin with sulphuric acid (cf. Gordon, Martin & Synge, 1943) under conditions that do not lead to destruction of *O*-methylethanolamine did not liberate any volatile amino compound. The non-volatile residue from such a hydrolysis contained only 39 % of the original methoxyl content of the methylated gramicidin, and chromatography on paper failed to reveal any trace of *O*-methylethanolamine. Clearly the methoxyl groups in methylated gramicidin are much more acid-labile than would be expected were they present in the ether linkage $—CH_2.CH_2.OCH_3$.

Table 1. *Relation of ethanolamine content to number of hydroxyl groups of gramicidin*

Group introduced	g. residues/10^4 g. original gramicidin	Reference
Acetyl	8·2	Hotchkiss (1941)
Acetyl	6	Fraenkel-Conrat *et al.* (1947)
Succinyl	6·6	Olcott *et al.* (1946)
Sulphuric ester	5·9	Reitz *et al.* (1946)
Phosphoric ester	6·1	Ferrel *et al.* (1948)
Methoxyl	5·4	This paper
Ethanolamine liberated in acid hydrolysis (after correction for estimated destruction)	6·1	Synge (1945)

A moderately plausible alternative would be to suppose that the glycine and ethanolamine found in roughly equimolecular proportions in acid hydrolysates of gramicidin (Synge, 1945) arise by a sort of Cannizzarro reaction from residues of aminoacetaldehyde present in the intact gramicidin (cf. Trier, 1911). These could be conceivably linked in a normal peptide chain thus:

$$—NH.CHR.CONH.CH_2.\underset{\displaystyle OH}{\underset{|}{CH}}.NH.CHR.CO—$$

giving a site for substitution of acid-labile *O*-methyl groups. We synthesized a model compound in which aminoacetaldehyde residues occurred in peptide linkage, namely the diethylacetal of carbobenzyloxy-L-valylaminoacetaldehyde. On treating this compound or the parent aminoacetal with hot acid neither glycine nor ethanolamine could be found among the reaction products. We were accordingly less inclined to regard the ethanolamine of gramicidin hydrolysates as an artifact, and endeavoured to obtain products by partial hydrolysis of gramicidin that might throw light on the mode of linkage of its precursor residues.

Partial hydrolysis studies

It was felt likely that the bonds linking ethanolamine or its precursor in the gramicidin molecule would be more labile to hydrolysis than normal peptide bonds, and therefore a study of partial hydrolysates should give some information on the mode of incorporation of ethanolamine.

It was found that gramicidin was surprisingly resistant to ethanolic alkali; no titratable groups were detected after treatment with 0·08 N-ethanolic sodium hydroxide at 37° for periods up to 1 week.

Acid hydrolysis using 10 N-hydrochloric acid in dioxan was found to be more effective—titration of the hydrolysate showed that 0·5 equivalents of acidic groups and 0·75 equivalents of basic groups/3800 g. (the most probable molecular weight of gramicidin given by X-ray analysis, see Hodgkin, 1950) were liberated after 16 hr. at 20°. Increasing the time of hydrolysis to 24 hr. liberated 1·1 equivalents of acidic groups and 1·75 equivalents of basic groups/3800 g. (Fig. 3).

The low-temperature hydrolysis of gramicidin using aqueous dioxan-10 N-hydrochloric acid mixtures was also studied and found to give a similar result after 9 days at 2° to that given by hydrolysis for 1 day at 20°. The relatively more rapid liberation of basic groups points to the presence of basic peptides that would be expected to be either *O*-acyl or *N*-acyl derivatives of ethanolamine (as no basic amino-acids are present in gramicidin). Such basic peptides were also found by Synge (1945) after 2 days' hydrolysis at 37° in 10 N-aqueous hydrochloric acid-acetic acid (1:1, v/v).

It was noticed that whenever these hydrolysates of gramicidin, dissolved in aqueous ethanol, were made more alkaline than pH 4, a copious white precipitate formed that would not dissolve at any pH above 4. Chromatography on paper showed that this precipitate differed from the material remaining in solution. Both low-temperature and room-temperature hydrolyses gave rise to this insoluble peptide material which, under the conditions used, invariably accounted for at least half of the original gramicidin. Two fractions were thus obtained by adjusting the hydrolysate to pH 6, denoted ethanol-soluble peptides and ethanol-insoluble peptides respectively. Substitution with the dinitrophenyl group indicated that both these peptide fractions contained tryptophan as the only amino-acid with an unsubstituted amino group. It does not seem previously to have been noted that DNP-tryptophan is unstable to acid hydrolysis and breaks down to give dinitroaniline and a yellow product which runs at the same rate as DNP-tryptophan on silica-gel chromatograms, but no longer gives any colour with the Ehrlich reagent.

Further fractionation of the ethanol-soluble peptides was carried out by electrophoresis in an ethanolic gel of silicic acid at pH 6. Two diffuse bands were obtained both moving towards the cathode. The fastest band consisted of tryptophan-free peptides giving a strong colour with ninhydrin and the slower band consisted of tryptophan-rich peptides that gave only a weak colour with ninhydrin. Poor recoveries of the peptides from the gel prevented this technique being used on a larger scale for the isolation of the different peptide fractions.

Chromatography on starch was found to be a more efficient method of fractionation of the ethanol-soluble peptides. By use of the solvent system *cyclo*hexane-*iso*propanol-water, gramicidin and gramicidin-like peptides (fraction 1) could be rapidly eluted from the column. Successive elution with ethanol and 60 % (v/v) aqueous ethanol gave two further fractions (fractions 2 and 3 respectively).

All these fractions were run on paper chromatograms using the *cyclo*hexane-*iso*propanol system. Fraction 1 contained apparently unchanged gramicidin and tryptophan-rich peptides, fraction 2 consisted of long-chain peptides containing tryptophan and giving a weak colour with ninhydrin and fraction 3 consisted of shorter peptides containing no tryptophan but giving a strong colour with ninhydrin. The marked inhomogeneity of each of these fractions was demonstrated by the paper chromatograms shown in Figs. 4 and 5.

The partial acid hydrolysis of the ethanol-insoluble peptide material was next studied, as it was felt likely that this represented an initial hydrolysis product of gramicidin. Hydrolysis with an aqueous dioxan-hydrochloric acid mixture showed that short-chain peptides were produced; these ran at the same rate on paper chromatograms as the short-chain peptides in fraction 3. It is concluded that peptides arise in the following stages: gramicidin → ethanol-insoluble peptide material → short-chain peptides.

Further analytical work was carried out on the fractions obtained from the starch column, each fraction being analysed for tryptophan and ethanolamine. The results are summarized in Table 2. It is clear that the material in fraction 3 consists of short-chain peptides rich in ethanolamine but containing no tryptophan. A similar fraction was obtained ionophoretically by Synge (1945) from partial hydrolysates of gramicidin. A qualitative amino-acid analysis, carried out on complete hydrolysates of each fraction by paper chromatography, showed that glycine, alanine, valine, leucine and tryptophan were present in all fractions except fraction 3 which contained no tryptophan and only a small amount of leucine.

The mixed peptides of ethanolamine contained in fraction 3 might be of two types: (*a*) *O*-acyl derivatives of ethanolamine: $RCO.OCH_2CH_2NH_2$, or (*b*) *N*-acyl derivatives of ethanolamine:

$$RCO.NHCH_2CH_2OH.$$

It is known that *O*-acyl derivatives of β-hydroxy-amino compounds rearrange spontaneously in alkaline solution to give *N*-acyl derivatives, the reverse change in acid solution being very much slower (Bergmann & Brand, 1923; Bergmann, Brand & Weinmann, 1923; Phillips & Baltzly, 1947):

$$RCO.OCH_2.CH_2NH_2 \xrightarrow{OH^-} RCO.NHCH_2.CH_2OH.$$

Titration of an amino-acid ester of ethanolamine would therefore show loss of one basic group on back titrating from an alkaline pH. Loss of basic groups on titration thus provides a means of detecting the presence of amino-acid esters of ethanolamine in gramicidin hydrolysates. This loss of basic groups in alkaline solution explains the failure to detect any free amino groups of ethanolamine in gramicidin hydrolysates by the FDNB technique, as the reaction was carried out under mildly alkaline conditions. The failure of the tryptophan-free material of fraction 3 to yield any DNP-amino-acid, despite its high amino N content, could be attributed to destruction of DNP-glycine under the hydrolysis conditions, or to other yet unknown causes.

Back titration of a low-temperature partial hydrolysate of gramicidin, prepared as described earlier, showed the expected loss of basic groups (Fig. 6). Similar titrations carried out on the fractions obtained from such a hydrolysate show that with the exception of the insoluble peptide fraction, all showed some fall in basic groups on treatment with

alkali. In Table 3 are shown the figures obtained from the titration curves and comparative figures obtained by measurement of free amino groups by the Van Slyke technique. The agreement between the two sets of figures is not good, the reason probably being inaccuracy in estimation of basic groups by the titration procedure owing to the difficulty in correcting the curves for the effects of the solvent on the titration. Nevertheless, the trend of the figures is in the same direction. A rather smaller loss of amino groups in fraction 3 than would be expected on the basis of its ethanolamine content might be due either to an insufficient reaction time being given in the Van Slyke apparatus or to ion-exchange effects on the column used for the fractionation causing rearrangement of the amino-acid esters of ethanolamine.

It is reasonable to assume from this evidence and that of Synge (1945) that the partial acid hydrolysis of gramicidin leads to the production of ethanolamine esterified with peptides of glycine, alanine, valine and leucine, together with long-chain peptides having tryptophan as the end residue carrying a free amino group.

Conclusions

Any structures put forward must account for the following facts:

(i) The only substances so far found in acid hydrolysates of gramicidin are the monoamino-monocarboxylic acids, glycine, alanine, valine, leucine, tryptophan, phenylalanine and tyrosine and the base ethanolamine. These account for all or nearly all of the carbon and nitrogen of gramicidin.

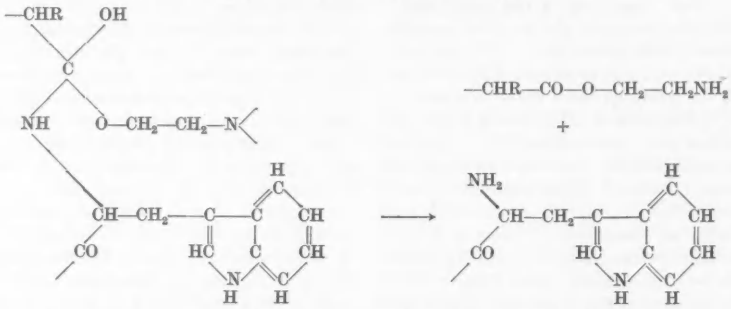
(ii) The free hydroxyl groups of gramicidin are not the primary alcoholic groups of ethanolamine residues.

(iii) The intact molecule possesses no free amino or carboxylic groups.

(iv) No volatile or non-volatile fatty acids or other ether-extractable carboxylic acids are present.

(v) No carbon dioxide is evolved during acid hydrolysis of gramicidin, suggesting that no ureido structure is present.

It is tentatively suggested that the ethanolamine residue is incorporated as such in the gramicidin molecule, being linked with a peptide bond by its hydroxyl group and linked with either an indole group of tryptophan or an enolized peptide bond by its amino group:



Methylation of the free hydroxyl group of the postulated structure would lead to formation of an acid-labile methoxyl compound. This structure is similar to that put forward by Phillips & Baltzly (1947) to explain the *O*-acyl rearrangement of esters of ethanolamine, and by Isbell & Frush (1949) to explain rearrangements of sugar acetates with ammonia.

EXPERIMENTAL

Materials

Melting points are given uncorrected. Optical rotations were measured in a 0·5 dm. tube. C, H and N (Dumas) determinations were by Drs Weiler and Strauss, Oxford.

Gramicidin. Commercial tyrothricin (Wallerstein Laboratories Inc., New York, Batch L30–13941) was extracted with a mixture of equal volumes of acetone and ether as described by Hotchkiss & Dubos (1941). The product obtained was repeatedly crystallized from dry acetone until the rotation was constant: $[\alpha]_D^{20°} + 3°$ in 95 % (v/v) aqueous ethanol (*c*, 0·17).

β-Methoxyethylamine was prepared by two methods, both simpler than that described by Traube & Peiser (1920). (*a*) β-Methoxyethanol (0·5 g.mol.) was treated with PBr_3 as described by Karvonen (1912) and the mixture was distilled. The fraction boiling up to 140° was collected in water, the oil separated, dried over $CaCl_2$ and fractionally redistilled. The fraction boiling over the range 102–104° was collected giving β-methoxyethyl bromide in 60 % yield (21 g.). This was refluxed with potassium phthalimide (13 g.) on a bath at 120° for 4 hr. The solution was cooled, filtered, evaporated to dryness *in vacuo* and the residue crystallized from water, aqueous ethanol and finally from dioxan affording *β-phthalimidoethyl methyl ether* (2 g.) as colourless needles, m.p. 147–148°. (Found: N (Kjeldahl) 6·0; OCH_3, 16·0. $C_{11}H_{11}O_3N$ requires N, 6·8; OCH_3, 15·1 %.) The β-phthalimidoethyl methyl ether was then hydrolysed with hot 6 N-NaOH and the liberated β-methoxyethylamine distilled and collected in water. The aqueous solution of the amine was titrated to neutrality with 0·1 N-acetic acid and made up to 50 ml. This solution of the amine acetate was analysed for

methoxyl content and N (Kjeldahl). The concentration of amine acetate given by the methoxyl estimation was 3·8 mg./ml. and by total nitrogen content 4·0 mg./ml. The identity of the amine was confirmed by preparation of the *N*-DNP-derivative, which was identical with the product described in (*b*) below.

(*b*) *N*-DNP-ethanolamine (3 g.) (for preparation see below) was dissolved in 20 ml. of dioxan and treated with a solution of 1 g. of dimethyl sulphate in 10 ml. of dioxan, 10 ml. of 10 N-NaOH were added and the two-phase system formed was shaken repeatedly and then left for 3 hr. at 37°. By the end of this time crystals had appeared in the dioxan layer. This layer was separated, evaporated to dryness *in vacuo* and the resulting oil crystallized from ethanol-acetone and then from $CHCl_3$ to give yellow prisms of *N-DNP-β-methoxyethylamine* (2·5 g.), m.p. 145·5°. (Found: C, 44·7; H, 4·6; N, 17·4. $C_9H_{11}O_5N_3$ requires C, 44·8; H, 4·6; N, 17·4 %.)

The product (2·5 g.) was refluxed with 20 ml. of 10 N-NaOH until dissolution was complete. The solution was distilled and the distillate titrated to neutrality with 0·1 N-acetic acid and made up to 50 ml. This solution was analysed for methoxyl content. The amine concentration from the titration was 5·5 mg./ml. and from the methoxyl estimation 5·3 mg./ml. The amine was identified by treating 2 ml. of the amine acetate solution with 80 mg. of fluorodinitrobenzene (FDNB) in 2 ml. of ethanol in the presence of 100 mg. of $NaHCO_3$. On standing, fine yellow prisms separated, which after crystallization from aqueous ethanol, had m.p. 144°. The mixed m.p. with an authentic sample of *N*-DNP-*β*-methoxyethylamine was identical.

N-2:4-*Dinitrophenylethanolamine.* Ethanolamine (0·01 g.mol.) was heated in ethanolic solution with 1-chloro-2:4-dinitrobenzene (0·005 g.mol.) for 3 hr. The solution was taken to dryness *in vacuo* and the residue crystallized from ethyl acetate-light petroleum to give orange-yellow needles (900 mg.), m.p. 91°. Recrystallization from aqueous ethanol afforded *N*-DNP-ethanolamine as needles, m.p. 96°. Waldkötter (1938) gives the m.p. as 90°. (Found: C, 42·9; H, 3·9; N, 18·3. Calc. for $C_8H_9O_5N_3$: C, 42·3; H, 3·9; N, 18·5 %.)

DNP-L-Tryptophan. L-Tryptophan (55 mg.) was dissolved in water (2 ml.) together with $NaHCO_3$ (200 mg.). FDNB (93 mg.) in ethanol (3 ml.) was added and the solution shaken for 3 hr. The ethanol was removed *in vacuo*, water was added and the solution was extracted three times with ether. The aqueous layer was acidified and the oily precipitate was crystallized from aqueous methanol to give DNP-L-tryptophan (70 mg.), m.p. 178° (decomp.). Porter & Sanger (1948) give m.p. 175°. (Found: C, 55·2; H, 3·2. Calc. for $C_{17}H_{15}O_6N_4$: C, 55·0; H, 3·8 %.)

Carbobenzyloxy-L-valylaminoacetal. Carbobenzyloxy-L-valyl chloride (Synge, 1948) prepared from 0·39 g. of carbobenzyloxy-L-valine in 4 ml. of ether after washing with ice water was allowed to react with 0·16 ml. of the diethylacetal of aminoacetaldehyde. On addition of saturated aqueous $KHCO_3$ solution and shaking *carbobenzyloxy-L-valylaminodiethylacetal* crystallized in the ether layer, was filtered off, washed with ether and dried (130 mg.). On recrystallization from ether m.p. was unchanged (133–134°). $[\alpha]_D^{15°} - 28°$ in ethanol (*c*, 1·5). (Found: C, 62·4; H, 8·4; N (Kjeldahl) 7·2. $C_{19}H_{30}O_5N_2$ requires C, 62·3; H, 8·2; N, 7·6 %.)

Analytical methods

Methoxyl determinations were carried out by the micro-Zeisel procedure using the apparatus of Steyermark (1948) modified in one respect. The reaction flask of the apparatus, instead of being sealed on was connected by a ground-glass joint so that the flask could be removed and solutions whose methoxyl content was required could then be evaporated to dryness *in situ*. The volumetric procedure (Grant, 1945) was preferred to the gravimetric estimation of silver iodide used by Steyermark. The method was standardized by means of pure vanillin; consistent recoveries of 95–97 % were obtained.

Kjeldahl nitrogen determinations on materials containing tryptophan residues were made after preliminary treatment with red P and HI according to Friedrich (see Grant, 1945; Hotchkiss & Dubos, 1941).

Tryptophan. The colorimetric procedure of May & Rose (1922) was employed. A series of standards were prepared using a pure sample of acetyl-L-tryptophan. The tubes were allowed to stand for 24 hr. at 37° followed by 24 hr. at room temperature in the dark to develop the full colour before readings were taken.

Ethanolamine. Each fraction was hydrolysed in a sealed evacuated tube using a mixture consisting of glacial acetic acid (1 vol.) and 5 N-HCl (4 vol.) for 24 hr. at 125°. A determination of the ethanolamine content of each fraction was carried out in Conway units by determining the ammonia liberated by periodate oxidation as described by Synge (1945).

Acid-base titration of gramicidin

In aqueous ethanol. Gramicidin (40 mg.) was dissolved in 2 ml. of 60 % (v/v) aqueous ethanol and titrated over the range pH 2–12 with 0·08 N-HCl and 0·08 N-NaOH using a glass electrode. The titration curve obtained was not significantly different from that given by titration of the solvent alone (Fig. 1).

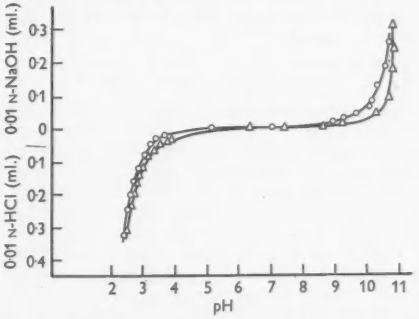


Fig. 1. The titration of gramicidin in 60 % (v/v) aqueous ethanol. △, Solvent only; ○, solvent + 40 mg. gramicidin.

In glacial acetic acid. Gramicidin (40 mg.) was titrated in glacial acetic acid with perchloric acid using crystal violet as indicator, by the technique of Nadeau & Branchen (1935). Despite a slight muddiness of the end point due to the presence of a small amount of water, a negligible amount of perchloric acid was necessary to make the solution acid.

Treatment of gramicidin with FDNB

Gramicidin (20 mg.) in 2 ml. of 60 % (v/v) aqueous ethanol was treated with 10 mg. of FDNB in 1 ml. of ethanol in the presence of 100 mg. of $NaHCO_3$ at 20°. After standing for

2 hr. the ethanol was removed *in vacuo* and the pale-yellow solution acidified with HCl. The colour of the solution disappeared and no coloured DNP derivative of gramicidin could be obtained.

Electrophoretic behaviour of gramicidin

The aqueous gels of silicic acid used by Consden, Gordon & Martin (1946) in their ionophoretic studies cannot be used for water-insoluble materials such as gramicidin. Ethanolic gels were prepared as follows and used instead. A mixture of ethyl silicate (Albright and Wilson Ltd., Oldbury) (100 ml.), ethanol (50 ml.), water (25 ml.) and 5 % (w/v) aqueous HCl (1 ml.) was shaken until a homogeneous system was obtained. This solution was stable for periods up to 3 weeks. When diluted (1:10) with 0·5 M-ammonium acetate in ethanol the solution gave a firm gel after 30–60 min. having pH 5–6. 120 ml. of gel were set in a trough 30 × 15 cm.

The brass cathode of the cell was bathed continuously with 0·5 M-ethanolic ammonium acetate kept acid by the addition of glacial acetic acid (final concentration 0·1 M). The carbon anode was bathed with 0·5 M-ethanolic ammonium acetate. The starting gutter in which the solution of gramicidin in the gel solution was set was displaced off-centre towards the anode. When a potential of 28 V./cm. was applied, the gel passed a current of 70 mA. The gel was kept cool by a water jacket on the bottom of the apparatus. The direction of electroendosmotic flow was shown to be towards the cathode by using a solution of *N*-DNP-ethanolamine in the gutter; the yellow band moved slowly towards the cathode. Gramicidin (10 mg.) was dissolved in 10 ml. of the gel-forming solution which was allowed to set in the gutter of the ionophoresis apparatus. A potential of 5 V./cm. was applied and a filter-paper print of the gel was taken after 5 hr. The paper was dried and sprayed with Ehrlich reagent. The gramicidin band was shown to have moved only 4 mm. towards the cathode.

Methylation of gramicidin

With dimethyl sulphate. Gramicidin (40 mg.) was dissolved in 3 ml. of dioxan and a solution of 0·5 g. of dimethyl sulphate in 3 ml. of dioxan was added. This solution was then treated with 1 ml. of saturated aqueous $Ba(OH)_2$, and the mixture shaken continuously for 1 hr. A further 1 ml. of the baryta was added and the shaking continued for 1 hr. The precipitated barium sulphate was then removed and the solution adjusted to pH 6 with a 0·01 N solution of H_2SO_4 in aqueous dioxan. The solution was filtered, evaporated to dryness *in vacuo*, the residue taken up in ethanol, precipitated with water and dried. The white powder thus obtained slowly turned purple on standing in air. This material contained 3·51 % OMe.

With methyl iodide. Gramicidin (40 mg.) was dissolved in 3 ml. of dioxan and 0·5 ml. of methyl iodide was added; 200 mg. of freshly prepared moist Ag_2O were added and the mixture warmed on the water bath to 60°. After 15 min. the solution was filtered, evaporated *in vacuo*, the residue was dissolved in ethanol, and precipitated with water; a stable white powder was formed. Methoxyl estimation of the product showed it to contain 1·68 % OMe. A number of other samples of gramicidin were treated in the same way and were shown to contain 1·54, 1·90, 1·75 and 1·74 % OMe corresponding to an average figure of 5·4 OMe groups/10^4 g. gramicidin. The nitrogen content (Kjeldahl-Friedrich) of one sample of gramicidin fell from 14·0 % N to 10·9 % on prolonged (35 min.) methylation with methyl iodide suggesting the possible occurrence of *N*-methylation, this big fall in percentage N being greater than could be accounted for by *O*-methylation.

Hydrolysis of methylated gramicidin

Gramicidin methylated by methyl iodide (200 mg.) was dissolved in 1·5 ml. of glacial acetic acid and 2 ml. of 10 N-H_2SO_4 were added. This mixture was heated in a sealed evacuated tube at 110–115° for 24 hr. The tube was opened, most of the acetic acid was removed *in vacuo* and the residual solution was transferred to the flask of a liquid-liquid extraction apparatus of the type used by Synge (1945) for the extraction of ethanolamine from a hydrolysate of gramicidin. Toluene (150 ml.) and 10 ml. of a saturated solution of $NaHCO_3$ were added, and the flask was then attached to the extraction apparatus and heated at such a rate that the toluene boiled vigorously. The condensed toluene dropped back through a funnel and passed upwards through a layer of water 12 cm. deep in the extraction tube. Any β-methoxyethylamine present would distil with the toluene and be extracted by the water layer. At the end of 3 hr. the extraction was considered complete and the aqueous layer in the apparatus was removed. The layer was titrated to neutrality with 0·001 N-HCl and required only four drops of acid. The solution was evaporated to dryness *in vacuo* below 40° and the residue was dissolved in water, and made up to 5 ml. Portions (1 ml.) of this solution were analysed for N and OMe. None of either was detected.

Stability of β-methoxyethylamine to acid hydrolysis. 1 ml. of an aqueous solution of β-methoxyethylamine acetate containing 5·69 mg. of the amine was treated in a sealed evacuated tube with glacial acetic acid (1·5 ml.) and 10 N-H_2SO_4 (2 ml.) for 24 hr. at 110–115°. The tube was opened and the contents were treated as described above for the methylated-gramicidin hydrolysate. The aqueous layer was removed from the extraction apparatus and was titrated with 0·012 N-HCl. The recovery of β-methoxyethylamine on the basis of the OMe content was 82 % and on the basis of the titration 90 %.

Counter-current distribution of a hydrolysate of methylated gramicidin

Methylated gramicidin (200 mg.) was hydrolysed in the acetic acid-H_2SO_4 mixture for 24 hr. at 125° and the hydrolysate was treated with saturated $Ba(OH)_2$ solution to remove excess H_2SO_4, the final pH being adjusted to 5. The filtered solution was then evaporated to dryness in the first tube of a 24-tube counter-current distribution apparatus. A 24-tube distribution was carried out using 30 ml. of the butanol phase and 15 ml. of the aqueous phase of the solvent system *n*-butanol (5 vol.), acetic acid (1 vol.) and water (4 vol.). Qualitative amino-acid analyses of the contents of each tube were carried out by means of *n*-butanol-water paper chromatograms (Fig. 2). Two spots that could not be accounted for showed up, the first running slower than glycine, and the second running slower than valine. A two-dimensional chromatogram (phenol-water-NH_3/collidine-water) of a small volume of the total hydrolysate showed the presence of at least ten spots, suggesting that the methylation with methyl iodide had caused *N*-methylation at the

peptide bonds, giving rise to *N*-methyl amino-acids on hydrolysis, as indicated by the drop in nitrogen content on methylation.

The fractions from the distribution were then combined into groups and each group was analysed for OMe content. The results are shown in Fig. 2. Only 39% of the original OMe content could be found in the hydrolysate of methylated gramicidin.

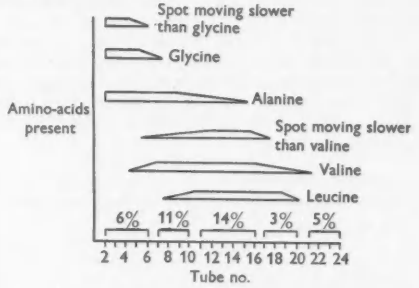


Fig. 2. 24-Tube counter-current distribution of acid hydrolysate of methylated gramicidin using *n*-butanol-acetic acid-water system. The amino-acids present in each fraction are indicated as well as the methoxyl content of grouped fractions. For details see text.

Behaviour of aminoacetaldehyde derivatives under acid hydrolysis conditions

Aminoacetal (*A*) (25 mg.) and 20 mg. carbobenzyloxy-L-valylaminoacetal (*B*) were heated in sealed evacuated tubes with 1·2 ml. 6 N-HCl and 0·3 ml. acetic acid for 27 hr. at 105°. The resulting solutions, which contained much brown 'humin' and had the smell characteristic of acid hydrolysates of protein, were evaporated to dryness *in vacuo* and taken up in water, one-fiftieth portions of the solutions (neutralized with NH_3 vapour) were chromatographed on Munktell OB filter paper using phenol-water. The brown pigment ran fast in each case; *A* gave, on heating after spraying with ninhydrin, two pink spots having R_F 0·25 and 0·75. *B* gave, without heating, a fast-moving spot corresponding with valine. However, in neither case was glycine or ethanolamine detected, addition of either or both of these substances giving rise to new spots on the chromatograms (R_F, 0·4–0·5).

Partial hydrolysis of gramicidin

Treatment of gramicidin with alkali. Three samples of the same batch of gramicidin (50 mg.) were each dissolved in 2 ml. of 0·08 M-NaOH in 60% (v/v) aqueous ethanol in a boiling tube. The tubes were evacuated and sealed. The first was allowed to stand at room temperature for 20 hr. and then opened and titrated *in situ* with 0·08 M-HCl in 60% (v/v) aqueous ethanol, using a glass electrode. The second tube was opened and titrated after 65 hr. at room temperature, and the third after 7 days at 37°. The curves obtained differed in no respect from blanks with the ethanolic NaOH.

Acid hydrolysis of gramicidin. Gramicidin (40 mg.) was dissolved in a mixture of dioxan (2 ml.), purified by the method of Oxford (1934), and 10 N-HCl (0·5 ml.). The tube was evacuated, sealed and kept overnight at room temperature. The liquid in the tube separated into two layers; the lower, dioxan-rich layer rapidly developed a pink colour and finally a deep-brown colour, whereas the upper layer remained colourless. After 16 hr. the tube was opened and the contents taken to dryness *in vacuo*, the temperature never being allowed to rise above 20°. The residue was dissolved in 60% (v/v) aqueous ethanol and titrated with 0·08 M-ethanolic NaOH to pH 9 using a glass electrode. The electrode was then changed for an 'Alki' glass electrode (Cambridge Instrument Company) and the titration continued up to pH 12. A flocculent white precipitate formed during the titration when the pH rose above 4 and would not dissolve at any pH above 4. The experiment was then carried out extending the time of hydrolysis to 24 hr. The titration curves obtained are shown in Fig. 3.

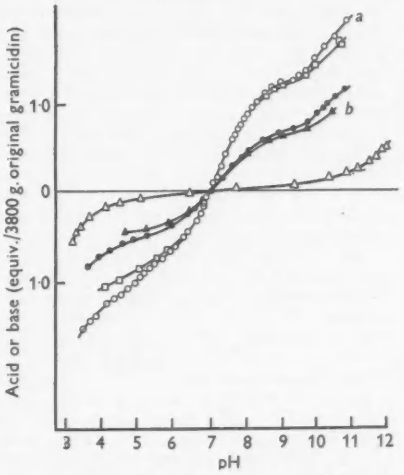


Fig. 3. Comparison of the rate of release of acidic and basic groups from gramicidin on partial hydrolysis by conc. HCl in dioxan solution. The curves are plotted to coincide at pH 7. For experimental details see text. △, Solvent blank titration. (*a*) 24 hr. hydrolysis at 20°; ○, experimental curve; □, curve corrected for solvent titration. (*b*) 16 hr. hydrolysis at 20°; ●, experimental curve; ▲, curve corrected for solvent titration.

Hydrolysis at low temperature using a mixture giving only one phase was then adopted. This was dioxan (10 vol.), water (3 vol.), 10 N-HCl (3 vol.). It was found that hydrolysis for 9 days at 2° gave a result similar to that obtained as above in 24 hr. at 20°. The low-temperature hydrolysis was used in all subsequent experiments.

End-group determinations on gramicidin hydrolysates

The terminal amino-acids, possessing a free amino group, present in these hydrolysates of gramicidin were determined by the method of Sanger (1945) involving the formation of the *N*-DNP derivatives and their separation on silica-gel columns. All solvent systems and columns used were first standardized by the use of pure preparations of DNP

amino-acids. The DNP-peptides were formed by treatment of solution of the peptide in 80 % (v/v) aqueous ethanol with excess FDNB in the presence of $NaHCO_3$ at 20°. After standing overnight the yellow solution was taken to dryness *in vacuo* and dissolved in water, acidified and extracted with $CHCl_3$. The DNP-peptides in $CHCl_3$ solution were precipitated by the addition of light petroleum, filtered and then hydrolysed *in vacuo* in a mixture of acetic acid (0·8 ml.) and 6 N-HCl (3·0 ml.) for 24 hr. at 125°. The hydrolysates were taken to dryness and then run on an ether-water silica-gel column to remove any residual mineral acid.

Fractions soluble in aqueous ethanol at pH's *above* 4. The DNP-amino-acids obtained from the ether-water column were run on the following series of columns (the solvent systems used were as described by Sanger (1945), except where otherwise specified):

(i) Ethanol-ligroin. Two bands were obtained, the first being a broad yellow band with $R = 0{\cdot}2$, and the second being brown with $R = 0{\cdot}1$. The faster band was not DNP-valine ($R = 0{\cdot}45$) or DNP-leucine ($R = 1{\cdot}0$). The two bands were collected separately and the material in the faster band was run on column (ii).

(ii) 5 % *iso*Propanol-*cyclo*hexane. One band was obtained ($R = 1{\cdot}0$). The band could not be DNP-glycine ($R = 0{\cdot}1$) or *N*-DNP-ethanolamine ($R = 0{\cdot}27$). The material in this band could therefore be either DNP-tryptophan or DNP-alanine. To test this point a mixed chromatogram with DNP-alanine was run on an ethanol-ligroin column. Two bands were obtained, the first having the rate of DNP-alanine ($R = 0{\cdot}25$) and the second $R = 0{\cdot}1$. This suggested that the unknown DNP-amino-acid was DNP-tryptophan, and was confirmed when a mixed column with DNP-tryptophan using the 5 % *iso*propanol-*cyclo*hexane mixture gave only one band with ($R = 0{\cdot}9$–$1{\cdot}0$). However, when the material was tested with the Ehrlich reagent no colour was given; DNP-tryptophan under the same conditions gave a green colour. However, tests on the stability of DNP-tryptophan to acid hydrolysis alone and in the presence of gramicidin under the conditions used for the DNP-peptides, showed that in both cases isolated material behaved in exactly the same manner as the DNP-amino-acid obtained from the DNP-peptides. The material in the slow brown band obtained from column (i) was found to revert to a yellow colour on standing and on running on an *iso*propanol-*cyclo*hexane column two bands were obtained the first with $R = 0{\cdot}86$, the second $R = 0{\cdot}30$. The material in the first band behaved in the same way on an ethanol-ligroin column as did the main band obtained from column (i). The material in the second band was shown not to be *N*-DNP-ethanolamine, DNP-glycine, DNP-valine, DNP-leucine, or DNP-tryptophan by comparison of its *R* value on ethanol-ligroin, or *iso*propanol-*cyclo*hexane columns. It was concluded that it might be dinitroaniline derived from the breakdown of DNP-tryptophan, as similar material was obtained after treating DNP-tryptophan with acid. The evidence therefore suggests that the products isolated from the DNP-peptides correspond with those resulting from DNP-tryptophan.

Fractions insoluble in aqueous ethanol above pH 4. The material obtained as above from the ether-water column was run on the following columns:

(i) 5 % *iso*Propanol-*cyclo*hexane. One broad band obtained ($R = 1$).

(ii) Ethanol-ligroin. One slow band obtained, moving at the same rate as DNP-tryptophan.

As only a small amount of material was available no further tests could be carried out. The aqueous residue from the hydrolysis of the DNP-peptide was run on a paper chromatogram using *n*-butanol-water. The only amino-acids found were glycine, alanine, valine and leucine, whereas the original ethanol-insoluble peptide hydrolysed under the same conditions contained the aliphatic amino-acids and also tryptophan. This suggests that the free amino group of this peptide is that of tryptophan.

No indication of the presence of any other DNP-amino-acids in the hydrolysed DNP-peptides could be obtained. It was hoped that *N*-DNP-ethanolamine would be present, and the solvent system methanol (2 vol.), water (1 vol.), $CHCl_3$ (5 vol.), and light petroleum (10 vol.) was elaborated in order to separate *N*-DNP-ethanolamine ($R = 0{\cdot}5$) from 2:4-dinitroaniline ($R = 0{\cdot}1$). No DNP-ethanolamine was, however, discovered.

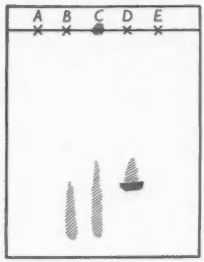


Fig. 4*a*.

Fig. 4*b*.

Fig. 5*a*.

Fig. 5*b*.

Figs. 4, 5. Paper chromatograms of fractions isolated from partial hydrolysates of gramicidin. Solvents: 60 % (v/v) aqueous ethanol (Fig. 4) and ethanol (Fig. 5) run to edge of diagram from starting lines shown: (*a*) sprayed with ninhydrin; (*b*) sprayed with Ehrlich's reagent. *A*, Gramicidin; *B* (band 1), *C* (band 2) and *D* (band 3) are fractions from starch columns; *E*, ethanol-insoluble peptide.

Fractionation of partly-hydrolysed gramicidin by electrophoresis

The peptides were dissolved in a small volume of the ethanolic gel already described and set in the gutter of the apparatus. A potential of 28 V./cm. was applied and the apparatus allowed to run for 5 hr. The position of the peptide bands in the gel was found by printing a filter-paper strip on the gel. One half of the paper strip was sprayed with the Ehrlich reagent in order to detect the position of the tryptophan-containing peptides, the other half was dried

and warmed *in vacuo* to remove ammonium acetate and then sprayed with ninhydrin, in order to detect the short-chain peptides. The faster, tryptophan-free band had moved 4 cm., the slower, tryptophan-rich band 2 cm. towards the cathode.

Peptide fractionation by partition chromatography on starch

Gramicidin (40 mg.) was partially hydrolysed at low temperature, and a solution of the hydrolysate was adjusted to pH 7 as described above. The insoluble peptide material (17 mg.) was removed by filtration and the filtrate was taken to dryness *in vacuo*. The residue was then run on a starch column (washed raw potato starch) using the solvent system water (1 vol.), *iso*propanol (3 vol.), *cyclo*hexane (7 vol.); the starch took up one-third of its weight of the aqueous phase.

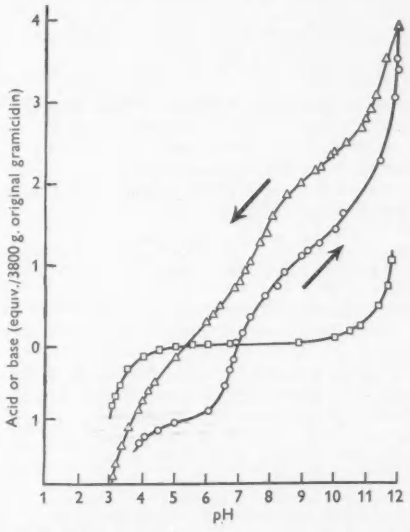


Fig. 6. Titration curves of partial hydrolysate of gramicidin showing loss of basic groups in back titration from pH 12. ○, Titration from pH 2 to 12; △, titration from pH 12 to 2; □, blank titration of solvent.

The column was examined in a dark room with an ultra-violet lamp. A fluorescent band ran rapidly through the column and was collected (fraction 1, 1 mg.), leaving an intensely fluorescent band at the top of the column that could be partly eluted by means of ethanol (fraction 2, 18·0 mg.) leaving a narrow fluorescent band at the top of the column. This band (fraction 3, 7 mg.) was finally eluted with 60 % (v/v) aqueous ethanol and was contaminated with the NaCl resulting from the original neutralization of the hydrolysate. The components present in each of these three fractions were investigated by the use of paper chromatograms using as solvents ethanol and 60 % (v/v) aqueous ethanol (Figs. 4 and 5).

Analysis of the fractions. Each fraction was analysed as follows: (1) tryptophan; (2) ethanolamine; (3) qualitative amino-acid analyses were carried out on hydrolysates of each fraction by using paper chromatograms with the solvent system *n*-butanol (5 vol.)-acetic acid-(1 vol.)-water (4 vol.); (4) the free amino N of each fraction was measured by the Van Slyke method (4 min. reaction time); (5) titration curves (Fig. 6) were obtained for the unfractionated acid hydrolysate of gramicidin and for each fraction by titrating first from pH 2 to 12 and then back to pH 7 in 60 % (v/v) aqueous ethanol and using glass electrodes with a stream of nitrogen gas as a stirrer, as described above (the titration mixtures were above pH 7 for about 10 min.); (6) the total N of each fraction was determined (Kjeldahl-Friedrich). The results are given in Tables 2 and 3. The results of the qualitative amino-acid analyses are given above (p. 111).

Table 2. *Ethanolamine and tryptophan content of fractions isolated from partial hydrolysates of gramicidin*

Fraction	Tryptophan (% by wt.)	Ethanolamine (% by wt.)
Gramicidin	23·8	3·7
Ethanol-insoluble peptides	21·0	4·8
Ethanol-soluble peptides:		
Fraction 1	18·0	3·7
Fraction 2	6·3	3·5
Fraction 3	0	21·4

SUMMARY

1. The absence of basic or acidic groups in gramicidin has been confirmed by titrations in aqueous ethanol and in glacial acetic acid, by its failure to form a coloured dinitrophenyl derivative and by its failure to move as a charged molecule in electrophoresis.

2. The presence of hydroxyl groups in the molecule has been confirmed by methylation with methyl iodide.

Table 3. *Change in amino-nitrogen content of fractions from partially hydrolysed gramicidin after treatment with alkali*

(Amino N as % of total N.)

	Before treatment		After treatment with alkali	
Fraction	Van Slyke amino N	Titration amino N	Van Slyke amino N	Titration amino N
Unfractionated hydrolysate	8·0	6·0	4·8	3·7
Ethanol-insoluble peptides	3·8	2·8	3·2	2·8
Ethanol-soluble peptides:				
Fraction 2	2·7	3·1	3·6	2·3
Fraction 3	21	—	15·8	—

3. Hydrolysis of such methylated gramicidin fails to liberate β-methoxyethylamine as would be expected if the methylated hydroxyl groups were those of ethanolamine residues.

4. The methoxyl groups of methylated gramicidin are shown to be acid-labile.

5. Residues of aminoacetaldehyde probably do not serve as precursors of the ethanolamine found in acid hydrolysates of gramicidin.

6. The relative rate of release of basic and acidic groups in the partial acid hydrolysis of gramicidin has been followed by means of titration curves. Gramicidin is not readily hydrolysed by alkali in the cold.

7. The free amino groups of the peptides produced on acid hydrolysis have been recognized as those of tryptophan residues. Dinitrophenyl-tryptophan is not stable to the acid hydrolysis conditions used.

8. Evidence is presented for the existence of amino-acid and peptide esters of ethanolamine in partial acid hydrolysates of gramicidin.

9. A partial structure for gramicidin is proposed.

10. The new compounds β-phthalimidoethyl methyl ether, *N*-2:4-dinitrophenyl-β-methoxyethylamine and carbobenzyloxy-L-valylaminoacetal have been synthesized, and improved procedures are described for preparing β-methoxyethylamine.

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The Role of Gastric Urease in Acid Secretion

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The experiments described in this paper were undertaken to throw light on the possible role of gastric urease in the secretion of hydrochloric acid by the stomach. Mathews (1925) first suggested that gastric hydrochloric acid might be produced by the reaction $NH_4Cl \rightarrow NH_3 + HCl$, and Luck (1924) showed that ammonia can be formed from urea by suspensions of ground dog gastric mucosa. Rigoni (1930), Martin (1932), Cardin (1933), Mann & Mann (1939), and Martinson (1950), all suggested that ammonium ions, derived from the enzymic hydrolysis of urea, form hydrogen ions according to the hypothesis of Mathews (1925).

Histochemical investigations by Linderstrøm-Lang & Ohlsen (1936) on dog mucosa, and Glick (1949*a*), on human stomachs, showed that the urease is localized in the oxyntic-cell region of the stomach. Glick (1949*a*) wrote 'the intensity of the (urease) activity appeared to be proportional to the acid-secretory response elicited by histamine', and put forward a tentative scheme for the formation of hydrochloric acid from the enzymic decomposition of urea. Luck & Seth (1925) first suggested that a possible function of the urease is to protect the mucosa from attack by acid.

All these hypotheses are based on qualitative evidence. In the present work quantitative data bearing on the hypotheses were obtained by comparing *in vitro* the rates of urea breakdown, ammonia production, respiration, and acid secretion, of isolated frog gastric mucosae. These experiments, and measurements of the urea content of frog gastric mucosae and mouse stomachs, show that gastric urease plays no direct role in the mechanism of acid secretion. Any neutralization of acid in our experiments is also excluded.

Part of this work has been communicated to the Biochemical Society (Davies & Kornberg, 1950*a*).

METHODS

Estimation of tissue urea. Large frogs (*Rana temporaria temporaria* L.) were pithed and the gastric mucosae rapidly dissected out and washed, according to the procedure of Davies (1948*a*). The wet mucosae were blotted and small pieces snipped off from the corpus regions for estimation of urease activity. The remaining tissue was rapidly weighed on a torsion balance and dropped into 5 ml. boiling water to destroy any gastric urease activity. Mice were killed by decapitation and the stomachs cut open, washed and treated as above.

After 4 min. boiling, the mixture was finely ground with a little acid-washed sand. Samples (1–2 ml.) of these mixtures were pipetted into the main compartments of Warburg vessels, together with 0·3 ml. 3 M-acetate buffer (pH 5·0) and 0·7 ml. water. The urea content was estimated manometrically at 38° by addition of 0·5 ml. jack bean urease solution. This enzyme solution was prepared by shaking 10 g. finely ground, sieved jack bean meal with 100 ml. water and 10 ml. 3 M-acetate buffer (pH 5·0) for 2 hr. After addition of two drops *iso*-octanol, the supernatant liquid was used as the enzyme source.

Estimation of tissue urease activity. The pieces of frog gastric mucosa, or of whole mouse stomach, obtained as above, were weighed and ground with a little sand and 5 vol. water. Samples (1 ml.) of the resultant suspensions, together with 3 ml. 0·3 M-acetate buffer (pH 5·0) were pipetted into the main compartments of double side-armed Warburg vessels, and, after equilibration at 25 or 38°, 0·4 ml. 0·275 M-urea solution was added from a side arm. The urease activity of the ground tissue was calculated as Q_{urea} from the rate of evolution of carbon dioxide. An hour after the addition of the urea, jack bean urease was added from the second side arm. The immediate evolution of CO_2 showed that any lack of effect with the ground tissue was due to an absence of urease activity and not to possible faults in technique.

Measurement of acid production by tied bags of frog gastric mucosa. The amounts and rates of acid secretion and respiration of histamine-stimulated isolated tied bags of frog gastric mucosa were measured manometrically at 25° by the method of Davies (1948*a*). In some of the experiments, urea (final concentration 2, 10 or 20 mM) was added to the tissues initially or during the course of the incubations (3–5 hr.).

Estimation of ammonia. The ammonia content of the incubation media, and the secretions formed in the tied bags of mucosa, were determined by the method of Conway (1947). The boric acid + indicator solution in the centre wells of the Conway units was quantitatively transferred to small tubes and titrated against 0·015 N-HCl run in from a Gilmont ultramicroburette. By this procedure the formation of less than 0·1 μmol. ammonia could have been detected.

Estimation of urea in media. The urea contents of the media were estimated manometrically with jack bean urease.

Units. All Q_X values are in μl. X/mg. dry wt. of tissue/hr., where 22·4 μl. X is equivalent to 1 μmol. X.

RESULTS

The urea content of frog gastric mucosa. The urea content of ten (pooled) samples of frog gastric mucosa is shown in Table 1. The average value was 0·039 μmol./mg. dry wt. This is 0·05 % wet wt., or

Table 1. *Urea content of frog gastric mucosa and of mouse stomach*

(Tissue rapidly washed, weighed and dropped into 5 ml. boiling water. Boiled for 4 min., ground with sand, and samples estimated manometrically for urea with jack bean urease in 0·3M-acetate buffer, pH 5·0.)

Tissue	No.	Total dry wt. (mg.)	Total urea content (μmol.)	Urea concentration (μmol./mg. dry wt.)
Frog gastric mucosa	3	58	2·4	0·041
	3	57	2·3	0·040
	2	58	2·2	0·0375
	2	61	2·35	0·0385
Mouse stomach	3	125	1·8	0·014
	2	90	1·5	0·0165

8·3 mM. Five samples of whole mouse stomach (Table 1) had an average urea content of 0·015 μmol./mg. dry wt., this being 0·019 % wet wt. or 3·2 mM.

The urease activity of frog gastric mucosa and mouse stomach. Estimations were made of the urease activity of thirty-seven ground frog gastric mucosae. Twenty-nine mucosae showed no trace of urease activity, and the Q_{urea} was therefore smaller than −0·02. Six of these samples were from mucosae which had been actively secreting acid *in vitro* with Q_{HCl} values up to 5·1. It may be that in these twenty-nine mucosae the urease was present but completely inhibited. This possibility cannot be excluded but is unlikely, because added jack bean urease was not inhibited. Urease activity was found in the remaining eight mucosae, with Q_{urea} values ranging from −1·15 to −1·50, average −1·38. It is not known whether these mucosae were capable of secreting acid, but this is likely as the experiments were carried out in spring (Bradford, Crane & Davies, 1950). No urease activity was found in any of eight unground open sheets of frog gastric mucosa. In contrast to these results, urease activity was found in all of ten mouse stomachs. The Q_{urea} values ranged from −3·6 to −6·8, average −5·2.

The effect of urea on ammonia formation by frog gastric mucosa. Since the ammonia produced by gastric urease activity is not preferentially incorporated into the proteins of the gastric mucosa (Davies & Kornberg, 1950*b*) a maximum figure for the urease activity can be derived from the ammonia produced during the course of an experiment. Assays were made of the ammonia content of the media in twenty-two experiments. Eighteen of these contained urea (10 mM), and in ten of these the mucosae were stimulated to secrete acid. Four of the mucosae were incubated in the absence of urea, and two of these secreted acid. In no case was ammonia detected in the medium. The secretions from ten mucosae were also assayed for ammonia. Six of the mucosae had been incubated in the presence of urea (10 mM) and five of them had secreted acid. Two of the remaining four mucosae incubated in the absence of urea had also secreted acid. No trace of ammonia was found in any of these secretions.

Control estimations showed that the method used was sensitive to less than 0·1 μmol. of ammonia. This means that the Q_{NH_3} was less than 0·01.

The recovery of added urea. In order to test the possibility that the urea was being metabolized by mechanisms not leading to the formation of ammonia, estimations were made of the urea content of twelve of the media after incubation. In all cases quantitative recovery of the urea was obtained within the experimental error, which was estimated as ±2 %

The effect of urea on the respiration and acid secretion of frog gastric mucosae. In more than a thousand experiments previously carried out in this laboratory, it had been found that isolated frog gastric mucosae can respire, and secrete acid, in the entire absence of external supplies of urea. In four experiments urea was added to final concentrations of 1·7 or 17 mM (0·01 or 0·1 %) 1 hr. after the beginning of incubation. These additions caused no changes in the rates of respiration during the course of the experiments (4 hr.).

The rates of acid secretion produced by isolated frog gastric mucosae showed large individual variations (Davies, 1948*a*; Bradford *et al.* 1950) both in the maximum rates and in the quantities of secretions produced. In thirty-eight experiments urea was added, to final concentrations of 2, 10 or 20 mM, either initially or during the course of incubations lasting 3 or 5 hr. These additions had no effect on the rates of acid secretion when secretion had already commenced. Normal secretory responses occurred in all cases in which histamine was added either simultaneously with, or after, addition of urea. Q_{HCl} values greater than 3 were regularly obtained. Some mucosae had Q_{HCl} values greater than 5, and more than 18 μmol. hydrochloric acid were secreted during the course of the experiment. The urea content of the mucosa was, however, only 0·5 μmol.

DISCUSSION

The role of gastric urease in acid secretion. The hydrogen ions formed by the stomach were thought by Mathews (1925) to be derived from ammonium ions, the ammonia being either absorbed or turned into 'ureido or carbamido compounds'. This view was accepted by Rigoni (1930), Martin (1932), Cardin (1933), Mann & Mann (1939), and Martinson (1950), who thought that gastric urease provided the ammonium ions from the hydrolysis of urea. Glick (1949*a*) considered the possibility that the ammonium ions were exchanged with hydrogen ions of the blood during acid secretion.

There are several lines of evidence against these views. Isolated gastric mucosa can secrete acid in the absence of added urea or ammonia, as has been shown for frogs and toads (Delrue, 1930; Gray, Adkison & Zelle, 1940; Teorell & Wersäll, 1945; Davies, 1946; Edwards & Edwards, 1948) and for the stomach or gastric mucosae of mice, rats, cats, dogs and polecats (Davies, 1946; Davenport & Jensen, 1948; Coy & Rehm, 1948; Patterson & Stetten, 1949; Bradford & Davies, 1950). The addition of urea does not lead to an increase in the amounts of acid secreted by isolated mouse stomachs (Davenport & Jensen, 1949) or frog gastric mucosae (Davies & Kornberg, 1950*a*; Glick, Zak & von Korff, 1950), and causes no detectable increase in respiration. In some species urea in sufficiently large amounts may cause inhibition of acid secretion (FitzGerald & Murphy, 1950; Glick *et al.* 1950).

The results described in this paper show that isolated frog gastric mucosae can secrete more than thirty times the amount of hydrochloric acid that could possibly be made from the tissue store of urea, even if the whole of this were available to the oxyntic cells. Isolated mouse stomach (Davies, 1948*b*; Davenport & Jensen, 1948, 1949) can secrete more than 1 μmol. hydrochloric acid/mg. dry weight in the absence of added urea. This is more than fifty times the amount of urea in the tissue.

Therefore the urea store of the gastric mucosa in both frogs and mice cannot account for the quantities of hydrochloric acid which are secreted *in vitro*.

The remaining possibility is that oxyntic cells possess a mechanism which can synthesize urea at one point and hydrolyse it at another. Such a system could lead to the formation of hydrochloric acid, the ultimate source of the hydrogen ions in this case being water (Davies, 1948*a*, 1951). There is no evidence for the occurrence of an urea-synthesizing mechanism in gastric mucosa (Krebs, 1934; FitzGerald & Murphy, 1948), and it has been shown that the location of the urease in the gastric mucosa is in cells other than oxyntic cells (Linderstrøm-Lang & Ohlsen, 1936; Ohlsen, 1941; Fossel, 1947). FitzGerald & Murphy (1949), and FitzGerald (1951), using the technique of Sen (1930), claim to have located the urease in the cells surrounding the oxyntic cells, although FitzGerald & Murphy (1950), using the same technique, suggest that the enzyme is actually present in the oxyntic cells. The staining technique used has been found to be unreliable (Glick, 1949*b*; Davies & Kornberg, unpublished experiments), as it can be made to demonstrate an apparent localization of urease in tissues definitely known not to have urease activity.

Further evidence against this possibility is the fact that in isolated gastric mucosa the rate of urea breakdown is usually many times less than the rate of acid secretion (Davenport & Jensen, 1949; Davies & Kornberg, 1950*a*; Glick *et al.* 1950). It has also been shown in the present paper that high rates of acid secretion can be observed in mucosae free from urease activity.

The evidence thus appears conclusive that gastric urease plays no direct role in the mechanism of acid secretion, a view now accepted by Glick and his co-workers (Glick *et al.* 1950).

The protective role of urease in gastric mucosa. It has frequently been suggested that gastric urease plays a role in protecting the mucosa from attack by acid and pepsin (Luck & Seth, 1925; Linderstrøm-Lang & Ohlsen, 1936; Ohlsen, 1941; FitzGerald, 1946; Fossel, 1947; FitzGerald & Murphy, 1950; Glick *et al.* 1950). The present results show that any neutralization of acid by ammonia was quantitatively insignificant with *Rana temporaria*. Ammonia was not detectable in either the secretions or the media after incubation of gastric mucosae in the presence or absence of urea for 3 or 5 hr. These results differ from those of Glick *et al.* (1950), who used *Rana pipiens* as experimental material and reported the presence of ammonia in secretions and media, in the average ratio of 4:1 in absence of added urea, and 2·7:1 in presence of 0·1 % urea. The ammonia concentrations reported by Glick and his co-workers are more than three hundred times greater than would have been detected in our experiments. This difference in results may be due to differences in the biological material used.

SUMMARY

1. The average urea content of frog gastric mucosa was found to be 0·039 μmol./mg. dry wt. (8·3 mM, wet wt.). In whole mouse stomach the average concentration was 0·015 μmol./mg. dry wt. (3·2 mM, wet wt.).

2. Urea (2, 10 or 20 mM) did not affect the rates of respiration or acid secretion of isolated frog gastric mucosae.

3. The urease activity of ground samples of frog gastric mucosa was low and very variable; only eight out of thirty-seven samples contained any

activity. Active urease preparations were obtained from all mouse stomachs examined.

4. None of thirty intact frog gastric mucosae showed any urease activity. The incubation media or secretions of resting or acid-secreting tied bags of mucosa contained no detectable quantities of ammonia, either in presence or absence of urea. Added urea was recovered quantitatively. Any acid-neutralizing role of urease in these experiments is thus excluded.

5. The amounts of acid secreted by isolated frog gastric mucosa or whole mouse stomach in absence of added urea were more than thirty times the urea content of the tissue.

6. Since there is no evidence that urea is synthesized by gastric mucosa, it follows that urea and urease play no direct or catalytic role in the mechanism of acid secretion.

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The Specificity of Cobra-venom Cholinesterase

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The high concentration of cholinesterase in cobra venom was first noted by Jynegar, Sehra, Mukerji & Chopra (1938). The enzyme is present in the venom of the Colubridae, though absent from that of other genera, notably the Viperidae (Jynegar *et al.* 1938; Ghosh, Dutt & Chowdhury, 1939; Ghosh, 1940; Zeller, 1949). Following the recognition of two types of cholinesterase, Zeller & Maritz (1945) and Zeller (1947) classified cobra-venom cholinesterase as an *e*-type or 'true' cholinesterase.

Bovet-Nitti (1947*a*, *b*) studied the esterase specificity of cobra venom. She reported that a number of aliphatic esters such as the glycol and glycerol acetins and ethyl acetate were hydrolysed, but that tributyrin, ethyl propionate, amyl acetate and the choline esters of the higher fatty acids were not hydrolysed. The rate of hydrolysis of the *n*-alkyl acetates fell rapidly with increasing chain length. She assumed that the same enzyme was responsible for the hydrolysis of both acetylcholine

and the aliphatic acetates and, aware that this assumption did not conform to the views then current of the specificity of 'true' cholinesterases, proposed the name 'acetylase' for the enzyme (Bovet-Nitti, 1947*b*). Zeller (1948*a*) found a proportionality between the rate of hydrolysis of β-chloroethyl acetate, ethyl chloroacetate and the cholinesterase content of a number of snake venoms, and on this and other evidence concluded that the cholinesterase was capable of hydrolysing these aliphatic esters. He considered that the cholinesterases of colubrid venom constituted a separate class (ophiocholinesterases or *c*-type cholinesterases) with similarities to both types of mammalian cholinesterases (Zeller, 1948*a*, *b*). Following the first publication of Adams & Whittaker (1948), in which they reported the ability of purified human erythrocyte cholinesterase to hydrolyse a wide range of aliphatic acetate and propionate esters, and Holton's (1948) finding that cobra-venom cholinesterase resembled the *e*-type cholinesterases in hydrolysing triacetin, Bodansky (1946), Zeller & Utz (1949) and McNaughton & Zeller (1949) found that hydrolysis of the haloesters used earlier was not, in fact, a property characteristic of snake-venom cholinesterases, but was one shared also by typical *e*-type cholinesterases.

There remains, therefore, only one obstacle to classifying colubrid venom cholinesterases with those of brain and erythrocytes, namely the rapid fall in the rate of hydrolysis of *n*-alkyl acetates with increasing chain length reported by Bovet-Nitti, which contrasts with the rise up to *n*-butyl acetate followed by a fall found with brain and erythrocyte cholinesterases (Adams & Whittaker, 1948; Adams, 1949; Whittaker, 1949; Mounter & Whittaker, 1950).

The present work has been undertaken to determine whether, in fact, cobra-venom cholinesterase resembles in its aliphatic specificity the cholinesterases of brain and erythrocyte and whether it is to be regarded as a typical aceto-cholinesterase (Sturge & Whittaker, 1950).

METHODS

Measurement of enzymic activity. Rates of hydrolysis were measured by the Warburg manometric technique (Ammon, 1933) in which acid liberated by the hydrolysis of the substrate displaces an equivalent amount of CO_2 from a $NaHCO_3$-CO_2 buffer at pH 7·4 and 38°. Each flask contained a total volume of 3 ml., all solutions being made up in 0·023 M-$NaHCO_3$, and after attachment to the manometers was gassed for 3 min. with 95% N_2+5% CO_2. In most experiments the enzyme was placed in the side bulb and the substrate in the main compartment. The amount of enzyme was adjusted to give a gas evolution of 50–300 μl. CO_2 in 30 min. The enzyme was made up freshly for each experiment by dissolving dried venom in 0·023 M-$NaHCO_3$, since it was found that solutions of the enzyme deteriorated rapidly even at 0°. Non-enzymic controls were included in all experiments and the appropriate corrections made.

Source of enzyme. The cobra venom was kindly provided by Dr H. Blaschko and was part of the sample used by Blaschko & Stiven (1950). The species of cobra from which this venom was obtained is not known. It was collected in Madras in 1899, but despite the age of the sample it still retains a high cholinesterase activity, liberating 875 μl. CO_2/mg. dried enzyme/hr. from 0·03 M-acetyl-β-methylcholine.

Substrates. Choline esters were obtained commercially, with the exception of propionylcholine, which was prepared in the laboratory as the perchlorate. The aliphatic esters were either commercial samples or were prepared in the laboratory and were all purified before use by washing, drying and distillation, the middle third of the distillate being collected. All esters satisfied the usual criteria of purity.

RESULTS

Hydrolysis of choline esters and water-soluble aliphatic esters

The p*S* initial rate curves were determined for acetylcholine, propionylcholine and acetyl-β-methylcholine. (p*S* is $-\log_{10}$ substrate concentration (M).) The results are shown in Fig. 1. The curves

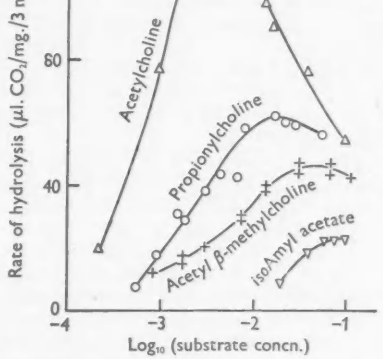

Fig. 1. Effect of substrate concentration on rate of hydrolysis of various substrates by cobra-venom cholinesterase. *iso*Amyl acetate emulsified with gum acacia before use.

are similar in form to those obtained by Augustinsson (1949) with the enzyme of *Naja naja* venom. At a concentration of 0·03 M, acetyl-β-methylcholine (which has an almost flat-topped curve) gives a nearly maximal rate of hydrolysis, and this substrate at this concentration was used as a standard for subsequent experiments.

Triacetin at a concentration of 0·05M gave a similar rate of hydrolysis to that obtained by Blaschko & Holton (1949), who used a different sample of cobra venom (cf. Augustinsson, 1949). Both choline ester and triacetin hydrolysis are eserine sensitive (see below). Butyrylcholine is hydrolysed at a very low rate; benzoylcholine and tributyrin are not hydrolysed. The relative rates of hydrolysis of these substances are given in Table 1.

Table 1. *Initial rates of hydrolysis of various esters by cobra-venom cholinesterase*

Substrate	Concn. (M)	Rate of hydrolysis (as % of rate for acetyl-β-methylcholine)
Acetyl-β-methylcholine chloride	0·03	100
Acetylcholine chloride	0·006	240
Propionylcholine perchlorate	0·006	115
Butyrylcholine chloride	0·006	2
Benzoylcholine chloride	0·006	0
Triacetin	0·05	52
Tributyrin	0·05	0

Experiments with sparingly soluble aliphatic esters without added stabilizing agent

In the first experiments with aliphatic esters the procedure used in previous specificity studies (cf. Mounter & Whittaker, 1950) was adopted, i.e. esters were added directly to the flasks (300 μmol./3 ml.) without previous emulsification, the flasks equilibrated for 10 min., the enzyme added from the side bulb and readings commenced after a further 5 min. Under these conditions it was found that ethyl and propyl acetates were hydrolysed, but that the acetates of higher alcohols were scarcely attacked (Table 2).

A possible explanation of these results was suggested by the work of Frazer & Stewart (1940), who found that oil-water emulsions of cobra venom were non-toxic when injected intravenously into rabbits. They attributed this to inactivation of the venom at the oil-water interface. The emulsion of sparingly soluble ester might have acted in the same way as the oily emulsions of Frazer & Stewart's experiments, bringing about a non-specific inactivation of the cholinesterase before it was able to attack the substrate appreciably. As it was not known from Frazer & Stewart's experiments whether, in fact, inactivation of cholinesterase had occurred concomitantly with the loss of toxicity in their preparations of venom, some experiments were performed to test this hypothesis.

Table 2. *Rates of hydrolysis of aliphatic esters (300 μmol./3 ml.) by cobra-venom cholinesterase expressed as percentage of acetyl-β-methylcholine rate: aliphatic esters present in part as separate phase*

Substrate	Rate
Ethyl acetate	6·5
Propyl acetate	4·6
Butyl acetate	2
Amyl acetate	1
*iso*Amyl acetate	2
Hexyl acetate	0
Amyl propionate	0

The inactivation of cobra-venom cholinesterase by oily emulsions and stabilization with gum acacia

A solution of cobra venom was shaken in a Mickle shaker for 5 min., alone or with paraffin, olive oil, diamyl ether or amyl butyrate (0·4 ml. of non-aqueous substance/10 ml. of venom solution); the last two substances were selected because of their chemical similarity to sparingly soluble substrates of aceto-cholinesterases. The cholinesterase activities of the samples were then compared with that of an untreated control using 0·03M-acetyl-β-methylcholine as substrate. Shaking for 5 min. was sufficient to produce a fine dispersion of the oily phase with formation of an extensive oil-water interface. Table 3 shows that in all samples containing an oily phase considerable inactivation had occurred and that inactivation was appreciable even when the enzyme was shaken in the absence of an oily phase. Experiments with a preparation of human erythrocytes, made by the method developed by Mounter & Whittaker (1950) for horse erythrocytes, are included for comparison. It will be seen that this cholinesterase, which hydrolyses sparingly soluble aliphatic esters without difficulty, is relatively stable under these conditions.

The most likely interpretation of these results is that advanced by Frazer & Stewart, namely, that the enzymes of cobra venom are (perhaps because of the relatively high concentration in which they

Table 3. *Inactivation of cholinesterases by shaking*

(Enzyme activities are percentage of unshaken control. Period of shaking, 5 min. in Mickle shaker, 0·4 ml. immiscible substance added/10 ml. enzyme solution.)

Source of cholinesterase	Shaken alone	Shaken with: Olive oil	Diamyl ether	Amyl butyrate	Paraffin
Cobra venom	48	20	15	12	38
	—	6	8	3	—
Erythrocyte	98	65	61	62	92

occur in the dried crude venom) peculiarly sensitive to inactivation by adsorption into interfacial surfaces. It seems unlikely that the results were due to traces of inhibitors present in the substances added, as these were of the purest obtainable grade and unrelated chemically, though the possibility that some of these compounds are weak competitive inhibitors cannot be excluded as an additional factor. As a further test some experiments were carried out with emulsions of varying degrees of dispersion. Water-immiscible substances were added to the enzyme in the main compartment of the Warburg flasks, directly or after emulsification in the Mickle shaker. In the flasks to which unemulsified water-immiscible substances had been added some degree of emulsification was produced by the normal agitation of the flasks in the Warburg bath. Other flasks contained 5% (w/v) gum acacia with the object of bringing about increased dispersion.

Taking first the results obtained in the absence of gum acacia it will be seen (Table 4, col. 2) that with the exception of paraffin prior emulsification increased the inactivation of the enzyme by the various aqueous immiscible substances tested. The results are in line with the surface inactivation hypothesis; paraffin, which rapidly separated out into a separate layer, whether previously emulsified or not, gave the least inactivation; olive oil and amyl butyrate, which gave fairly stable emulsions, gave the greatest inactivation, while the reduction in rate resulting from the somewhat finer emulsions obtained from the Mickle shaker was greater than when the substances were added directly to the bottles.

The results obtained in the presence of gum acacia showed, however, an important difference. In each case the inhibitory effect of the oily phase was greatly reduced; the gum appeared to stabilize the enzyme in addition to stabilizing the emulsion. Possibly the gum itself is absorbed at the interface, reducing the adsorption of the enzyme. Augustinsson's (1948) observation that gum acacia alone has a small potentiating effect on cholinesterases was incidentally confirmed (Table 4, cols. 2 and 3). The protection afforded is neither complete nor permanent; the rate of hydrolysis falls off more rapidly than does the control sample without added oil (Fig. 2).

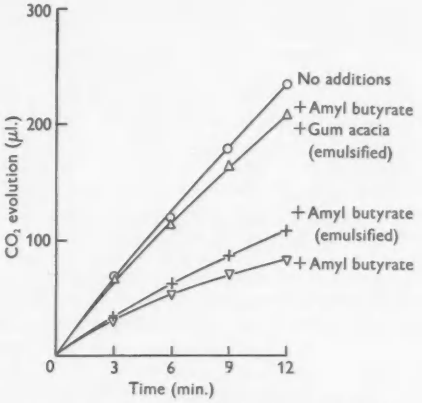

Fig. 2. Effect of *n*-amyl butyrate on hydrolysis of acetyl-β-methylcholine.

In addition to gum acacia, gelatin and egg albumin were found to protect cobra-venom cholinesterase against the effect of oily emulsions. Sparingly soluble aliphatic esters are hydrolysed in the presence of these substances at about the same rate as in the presence of gum acacia (Table 5). Gum acacia proved, however, to be the most convenient stabilizing agent for general use as egg albumin was denatured after some minutes at 38°,

Table 4. *Inactivation of cobra-venom cholinesterase by water immiscible substances and its protection by gum acacia*

(Substrate, acetyl-β-methylcholine (0·03 M); no. of experiments averaged shown in brackets.)

Aqueous immiscible substance added	Enzyme activity (% control without additions)		Protection afforded by gum acacia (%)
	Without gum acacia	With gum acacia (5%)	
None	100	109 (3)	—
Without previous emulsification:			
Olive oil	75 (2)	109 (2)	100
Amyl butyrate	42 (2)	96	88
Diamyl ether	95	100	92
Paraffin	90 (2)	—	—
With previous emulsification:			
Olive oil	9 (2)	68 (2)	63
Amyl butyrate	35	90	83
Diamyl ether	82	102	95
Paraffin	90	—	—

while gelatin was inconvenient to work with owing to the large change in viscosity between room and bath temperatures. All three protecting agents retarded somewhat the spontaneous loss of activity of venom cholinesterase solutions.

Table 5. *Hydrolysis of amyl acetates in presence of protecting substances*

(Rates expressed as percentage of rate with acetyl-β-methylcholine (0·03 M); concentration of amyl esters, 150 μmol./3 ml.)

Substrate	Gum acacia (5%)	Rate of hydrolysis in presence of: Egg albumin (5%)	Gelatin (2%)
*iso*Amyl acetate	40	53	43
n-Amyl acetate	25	33	28

Specificity studies with aliphatic esters in the presence of gum acacia

Specificity studies similar to those with other aceto-cholinesterases were now carried out in the presence of gum acacia, on the assumption that the gum would protect the enzyme against any non-specific inactivation by oily droplets of incompletely dissolved substrate, and that the rates of hydrolysis so obtained would give a truer picture of the specificity of the enzyme. The alternative, to work only with saturated aqueous solutions of aliphatic esters free from oily droplets, was rejected on account of the extremely low aqueous solubility, in the absence of the enzyme, of many of the esters which it was desired to investigate. As shown in Fig. 1, a total concentration of not less than about 150 μmol./3 ml. is necessary to ensure the attainment of a maximum rate of hydrolysis of *iso*amyl acetate, and an even higher concentration appears to be needed with triacetin (Augustinsson, 1949). The esters (150 μmol.) were added to the flasks after having been first emulsified with 5% (w/v) gum acacia. (Addition of substrates to a mixture of gum and enzyme gave slightly lower rates.) The results are shown in Figs. 3 and 4.

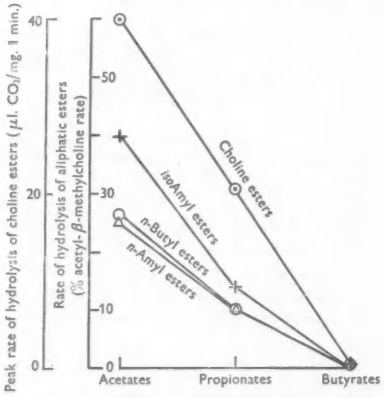

Fig. 3. Effect of acyl group size on hydrolysis of choline and aliphatic esters by cobra-venom cholinesterase.

It will be seen that the specificity pattern obtained is essentially the same as that of other aceto-cholinesterases (Adams, 1949; Whittaker, 1949; Mounter & Whittaker, 1950), the rate of hydrolysis of many esters which appear to be unattacked by the enzyme in the absence of gum acacia now being appreciable. Complete protection is probably not attained; thus the results were influenced somewhat by the amount of dispersion, and completely linear rates of hydrolysis were not obtained (Fig. 5). A satisfactory degree of reproducibility was, however, ensured by means of the following precautions. Acetyl-β-methylcholine and later *iso*amyl acetate were included in all experiments as controls. The preparation of the emulsion was carefully standardized, the concentration of enzyme increased to give a relatively high rate of hydrolysis, and a lower ester concentration was used than in previous specificity studies. Although detailed substrate concentration-initial velocity studies were not carried out for every substrate, a few experiments with higher substrate concentrations suggested

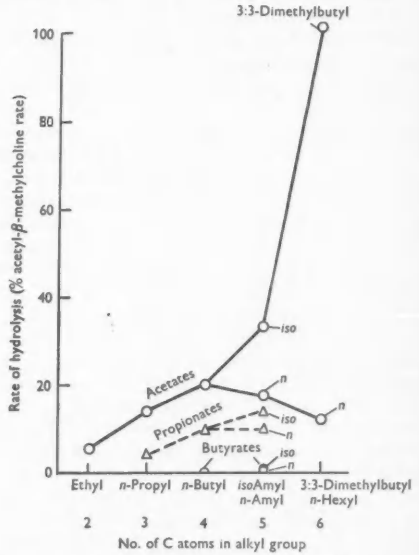

Fig. 4. Effect of size and configuration of alkyl group on hydrolysis of aliphatic esters by cobra-venom cholinesterase.

that this concentration was enough to saturate the enzyme. Manometer readings were taken at 3 min. intervals, corrected for non-enzymic hydrolysis, and the extrapolated initial rate taken. No individual result differed from the mean to a sufficient extent to alter the specificity pattern.

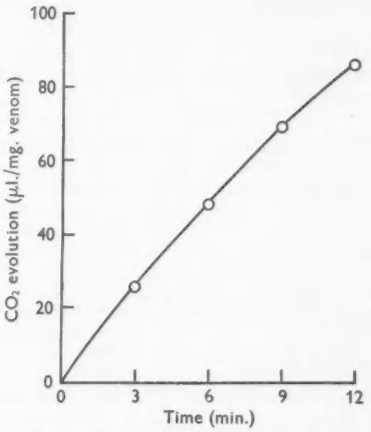

Fig. 5. Time course of hydrolysis of *iso*amyl acetate (150 μmol./3 ml.) emulsified in gum acacia (5 %) by cobra-venom cholinesterase.

Evidence for absence of other carboxylic esterases

Ester hydrolysis was completely inhibited by 10 μM-eserine (Table 6), indicating that the hydrolysis was in fact due to cholinesterase and not to other carboxylic esterases (Richter & Croft, 1942).

Table 6. *Inhibition of ester hydrolysis by eserine* (10 μM)

Substrate	Enzyme activity (μl. CO_2/ml./30 min.) Control	Control + eserine	Inhibition (%)
Aqueous solutions of esters:			
Acetyl-β-methylcholine*	1470	0	100
Ethyl acetate†	80	0	100
Emulsions of esters in gum acacia:			
Butyl acetate†	450	0	100
n-Amyl acetate†	570	0	100
iso-Amyl acetate†	770	18	97
iso-Amyl propionate†	145	0	100

* 0·03 M. † 150 μmol./3 ml.

DISCUSSION

The results presented above show that cobra-venom cholinesterase possesses the kinetics and specificity characteristic of aceto-cholinesterases. This extends to its behaviour towards aliphatic esters, the apparent inability of the crude venom to hydrolyse many aliphatic esters which are attacked by other aceto-cholinesterases having been traced to a non-specific inactivation of the enzyme which is brought about by oily substances generally, irrespective of whether they are potential substrates or not. The rates of hydrolysis obtained in the presence of gum acacia are believed to represent the true rates of hydrolysis of these substrates, since: (*a*) gum acacia does not appreciably affect (actually slightly raises) the hydrolysis of a 'normal' substrate like acetyl-β-methylcholine (Table 4); (*b*) identical rates are obtained with diverse stabilizing agents (Table 5); and (*c*) the rate of hydrolysis of aliphatic esters by erythrocyte cholinesterase is unaffected by prior emulsification with gum acacia (unpublished results).

To summarize these characteristics (cf. Whittaker 1951) acetyl- and propionyl-choline are hydrolysed, giving humped Murray-Haldane curves similar to those obtained with erythrocyte, brain and electric-organ cholinesterase (Augustinsson, 1949); acetyl-β-methylcholine is also hydrolysed but not butyryl- or benzoyl-choline. With aliphatic esters there is an increase in the rate of hydrolysis of acetates and propionates as the choline configuration is approached, acetates being hydrolysed more rapidly than the corresponding propionates; no butyrate is hydrolysed. An exact comparison of the present results with those of Bovet-Nitti (1947*a*, *b*) cannot be made, but they do not appear to be incompatible if, as seems likely, Bovet-Nitti's experiments were carried out without the addition of a stabilizing agent or with aqueous solutions of esters. Certain experimental results of Zeller and his co-workers are also in agreement with those obtained here, although his earlier conclusion that cobra-venom cholinesterases comprise a separate class of enzyme, i.e. do not fall into either of the two groups of 'true' or 'pseudo' cholinesterase (otherwise aceto- and butyro-cholinesterase), is now seen to have arisen from the use of too few aliphatic substrates and from the lack of comparisons with other cholinesterases.

It has been previously pointed out that variations in details are to be expected in the specificity pattern of individuals within the main cholinesterase classes. Thus pigeon-brain cholinesterase hydrolyses aliphatic esters relative to acetyl-β-methylcholine at about one-half to three-quarters of the values obtained with erythrocyte cholinesterases (Whittaker, 1949). With the cobra-venom cholinesterase the values are still lower; moreover, there is a sharper fall with increase in size of the acyl group from acetate to propionate.

The conclusion that cobra-venom cholinesterase is a typical aceto-cholinesterase, differing only in detail from other members of this class is of special

interest for several reasons. It is the first cholinesterase of cold-blooded animals to be so identified; it occurs in a secretion, and not, as do other members of the class, in intimate association with structural elements of the cell; it is present in high activity in venoms whose neurotoxic properties are much in evidence. Much further work on this and the many other enzymes present in this secretion will, however, be necessary before a clear understanding of their biological significance is attained.

SUMMARY

1. The hydrolysis of choline and non-choline esters by cobra venom has been studied: all hydrolysis is attributed to cholinesterase.

2. It is suggested that the enzyme is inactivated at interfaces giving an erroneous idea of the specificity pattern when sparingly soluble esters are used. The enzyme can, however, be protected by the addition of gum acacia or inert proteins.

3. The protected enzyme gives a specificity pattern similar to those found for other acetocholinesterases. Acetates are hydrolysed faster than the corresponding propionates; butyrates are not hydrolysed. The rate of hydrolysis of aliphatic esters increases as the acetylcholine configuration is approached, giving a maximum with 3:3-dimethylbutyl acetate.

I am greatly indebted to Dr V. P. Whittaker for his interest and advice in this work; also to Dr H. Blaschko for the gift of cobra venom and to the Medical Research Council for a personal grant.

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Observations on the Formation and Structure of Bacterial Cellulose

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Sisson & Clark (1933) and Sisson (1936), employing X-ray technique, studied cellulose produced from sugar by *Acetobacter xylinum*. Sisson's conclusions confirm and supplement earlier observations by Mark & Susich (1929), Hibbert & Barsha (1931) and Khouvine, Champetier & Sutra (1932). Later, bacterial cellulose produced from glucose by *A. xylinum* was examined by means of the electron microscope by Franz & Schiebold (1943), while Frey-Wyssling & Mühlethaler (1946) applied the same technique to the study of cellulose formed by *A. xylinoides*. Mühlethaler (1949) has continued these studies using cellulose formed by *A. xylinum* in a beer-sucrose medium.

Kaushal & Walker (1947, 1951) reported the ability of certain species of *Acetobacter* other than

A. xylinum and *A. xylinoides* to form cellulosic membranes when cultivated in specified carbohydrate media. In particular, a strain of *A. acetigenum* isolated in an East African vinegar brewery was noteworthy in this respect. Observations made, when membranes formed by *A. acetigenum* from a number of substrates were examined by X-rays and by the electron microscope, are now recorded.

EXPERIMENTAL AND RESULTS

X-ray examination of samples of cellulose formed by Acetobacter acetigenum *grown on different substrates*

The cultures employed were *A. acetigenum* (Henneberg) Bergey, National Collection of Type Cultures 5346, and two strains of the same species isolated from East African vinegar brews. They were grown on media consisting of the several carbohydrates or related substances (from 2 to 5 %, w/v) in yeast-water (prepared by boiling 75 g. of brewer's pressed yeast in 1 l. of water for 30 min.), or in the solution of nutrient inorganic salts recommended by Henneberg (1926) in which $(NH_4)_2SO_4$ is the source of nitrogen. Growth in the last-mentioned medium was enhanced by addition of corn-steep liquor (1 %, v/v) or of Witte peptone (0·2 %, w/v). The media were usually contained in large conical flasks which had wide necks or in the flat, circular, glass dishes originally devised for penicillin production (Clayton, Hunwicke, Hanes, Robinson & Andrews, 1944). Later, larger areas of cellulose were developed in a rectangular glass trough fitted with a flat glass cover which rested on a pad of sterile non-absorbent cotton wool to admit air. Each medium was inoculated from tube cultures prepared from the same liquid medium, the whole contents (10 ml.) of one tube, which had been inoculated from a platinum loop and incubated for 48 hr. at 25°, being employed to inoculate 200 ml. of solution in a flask. The depth of liquid in the flasks was usually from 1 to 1·5 cm. The surface pellicles were examined when the cultures had been developing for about 14 days at 25°, but in some cases incubation was conducted at room temperature (20–22°). Treatment with cold NaOH (2 %, w/v) for 10 hr., followed by washing with water, then with dilute acetic acid, and finally with distilled water for 12 hr., freed the cellulose from traces of protein, salts and other matter. The purified pellicles after drying at room temperature were obtained as very thin, almost colourless, films.

Usually these films were folded several times before X-ray examination, and the time of exposure varied from 4 to 6 hr. Table 1 shows the several substrates and the respective interplanary spacings in the cellulose from these substrates. These spacings were calculated by measuring the ring diameters as described by Clark (1940).

Since X-ray photographs of cellulose produced by the action of *A. xylinum* on glucose, on sucrose and on certain other substrates have already been published by Barsha & Hibbert (1934), we submit only our X-ray photographs of cellulose from cultures on media containing ethylene glycol, glycerol and maltose, respectively. These are reproduced as Figs. 1–3 and may be compared with the X-ray photograph of non-absorbent cotton (Fig. 4). In each photograph, *D*, the distance from the plate, was 3 cm. Non-absorbent cotton was used in mistake for absorbent cotton when preparing Fig. 4. The non-absorbent material contains 0·16 % (as Al_2O_3) of an aluminium compound added to confer non-absorptive properties, but this small quantity is insufficient to interfere with the typical X-ray photograph of cotton cellulose.

Rotation photograph of a cellulose membrane from a glycerol medium

Hitherto, the literature has not contained any reference to examination of bacterial cellulose by means of a rotation photograph, and to obtain evidence from this source we have photographed in a 19 cm. powder camera, a membrane (from a glycerol medium) 1·5 cm. in breadth and rolled into a cylinder of about 0·7 mm. in diameter. The results are given in Table 2 and the photograph is reproduced as Fig. 5. The photographs depicting the X-ray diffraction rings of bacterial cellulose and the data given in Tables 1 and 2 render it clear that the calculated interplanary spacings are in close agreement with those observed for cotton. The interplanar spacings of the three principal planes 101, 10$\bar{1}$, and 002, agree very closely with those calculated on the Meyer and Misch model of the

Table 1. *Interplanar spacings in cellulose from different substrates*

(Readings are measured in Ångström units.)

Ethylene glycol	Glycerol	Mannitol	Glucose (*a*)	Glucose (*b*)*	Fructose	Maltose	Non-absorbent cotton
6·18	6·12	6·12	6·12	6·12	6·12	6·18	6·20
5·45	5·45	5·45	5·45	5·48	5·48	5·45	5·45
4·35	4·40	4·35	4·40	4·35	4·35	4·40	4·35
3·95	3·92	3·95	3·92	3·95	3·92	3·95	3·95
3·20	—	3·15	—	3·15	3·20	3·25	3·20
—	2·95	2·90	2·95	2·95	2·95	2·95	—
2·70	2·68	2·68	2·66	2·68	2·66	2·68	2·70

* The values given under (*b*) in the glucose column were obtained by examination of a stretched membrane.

Table 2. *Data from rotation photograph of cellulose synthesized in a glycerol medium*

(Time of exposure, 36 hr. X-ray film corrected to standard length, 270·1 mm.)

Distance of arcs from one end, corrected for contraction (mm.)	log $\sin^2\theta$ from curve	log $\sin\theta$	$d=\lambda/2\sin\theta$
7·6	$\bar{2}$·204	$\bar{1}$·102	6·09
10·6	$\bar{2}$·311	$\bar{1}$·155	5·39
16·8	$\bar{2}$·493	$\bar{1}$·246	4·36
21·1	$\bar{2}$·584	$\bar{1}$·292	3·93
30·2	$\bar{2}$·766	$\bar{1}$·383	3·19
34·5	$\bar{2}$·842	$\bar{1}$·421	2·92
38·7	$\bar{2}$·909	$\bar{1}$·454	2·70

monoclinic unit cell of cellulose, namely 6·05, 5·45 and 3·95 A. respectively (compare Clark, 1940).

In one experiment (see Table 1, column 4, *b*) the purified membrane was folded and dried under tension before examination, and its X-ray diagram showed uniaxial preferred orientation in agreement with the observations of Sisson and others. Previous workers, employing *A. xylinum*, studied membranes derived usually from growth on glucose and on sucrose. From sucrose Eggert & Luft (1930) obtained a membrane which yielded an X-ray pattern similar to that given by cotton cellulose. Finally, it is necessary to add the qualification that while the essential agreement between the spacings observed by us for cellulose synthesized from various carbon sources would indicate that the unit cell is the same in all cases, this need not necessarily imply that the molecules within the cell, or their distribution, are identical in all cases.

Examination of bacterial cellulose by the electron microscope

Preparation of the membranes. The media were inoculated with liquid taken on a platinum loop from liquid cultures in malt wort which had been developing for about 48 hr. at 27°. Incubation was then carried out at room temperature (20–22°). Material was removed from the cultures for examination, in some cases as early as 18 hr. after inoculation. If such young cultures of *A. acetigenum* are observed carefully, it will be found that when cellulose first becomes visible to the naked eye it is not seen floating on the surface of the liquid but appears as a submerged and very fine mainly gelatinous membrane which has a diaphanous appearance. Later, this delicate structure rises to the surface, and on further development forms the characteristic pellicle. This early growth of material was taken up on a grid and was washed. In some cases the material was transferred first to a test tube, washed in the latter, and then taken up on the grid. After drying *in vacuo* the material was examined in the usual manner and Figs. 6–10 represent a selection of the electron micrographs which were prepared.

If *A. acetigenum* is grown in a test tube on a liquid medium containing glucose, or other suitable carbon source, together with a little kieselguhr (the latter being added for the purpose of producing turbidity when the tube is disturbed), after 10 days the cellulosic pellicle will be about 2 mm. thick and will adhere so firmly to the glass that the tube can be inverted and still retain its contents. We have observed that when this is done the kieselguhr sets up a turbidity which, however, does not extend downwards to what appears to be the undersurface of the pellicle, but stops along an interface which follows the line of the underside of the visible pellicle at a distance of about 2 mm. from the latter. Thus, between the turbid liquid and the opaque pellicle, there is revealed a zone or layer consisting of a transparent colourless gel which, otherwise, would escape observation. It would seem not unreasonable to suggest that this transparent layer consists either of lower polymer or of cellulose at a stage before crystallization has commenced.

Finally, Fig. 11, kindly prepared for us by Mr J. A. Lord, represents a sectional photograph of cellulose produced by *A. acetigenum* on a medium containing starch hydrolysate as source of carbon. After purification the specimen was photographed with an optical microscope-microcamera. The figure shows that during the formation of bacterial cellulose layering of fibrils takes place and, further, that the thickness of the fibrils appears to be of the same order, namely, 250 A., as is the thickness of many of those revealed by the electron microscope.

DISCUSSION

Franz & Schiebold's (1943) electron micrographs show cells of *A. xylinum* from 10,000 to 20,000 A. in length and from 3000 to 5000 A. in breadth. Surrounding these cells are submicroscopic strands of cellulose, the orientation of which is random, and the authors point out that these strands appear to be interwoven. Further, they state that the visible cellulose threads are ribbon-like, of length often 40 μ., with a minimum breadth of about 200 A. and thickness 100 A. Bands of up to 5000 A. in width and consisting of a number of narrower bands were also observed.

Frey-Wyssling & Mühlethaler's electron micrographs illustrate the crystallization of submicroscopic cellular strands from multimolecular cellulose films formed by *A. xylinoides*. These authors state that all the cellulose threads have the same diameter (200 A.) and the micrographs demonstrate the presence of 'dark threads embedded in a thin

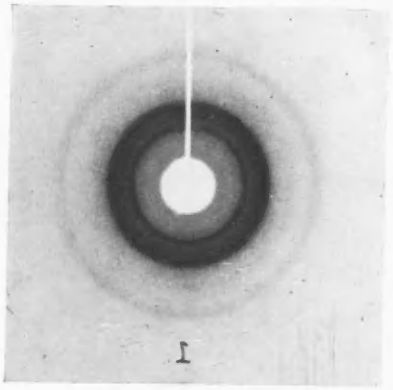


Fig. 1.

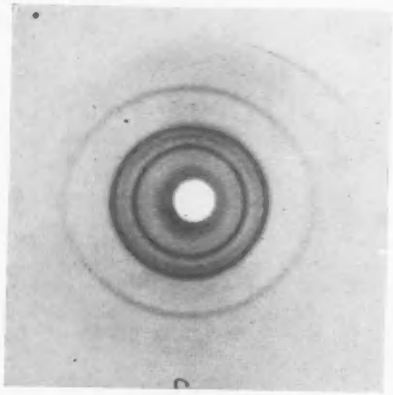


Fig. 2.

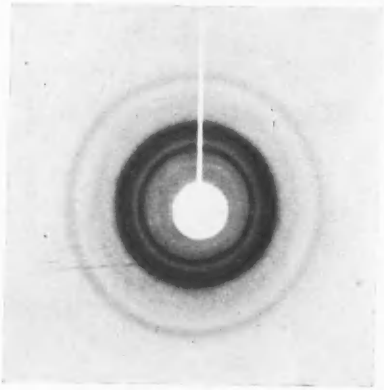


Fig. 3.

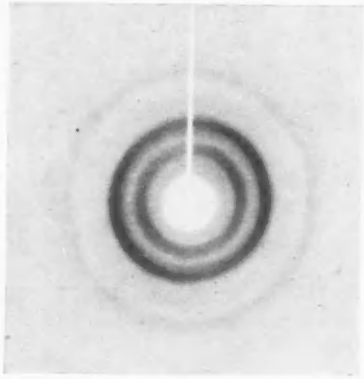
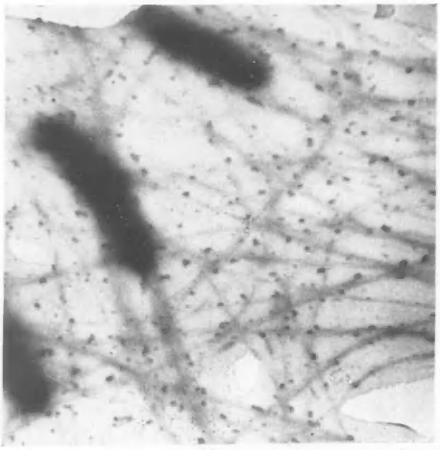


Fig. 4.

Fig. 5.



μ.

Fig. 6.

μ.

Fig. 8.

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Fig. 7.

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Fig. 9.

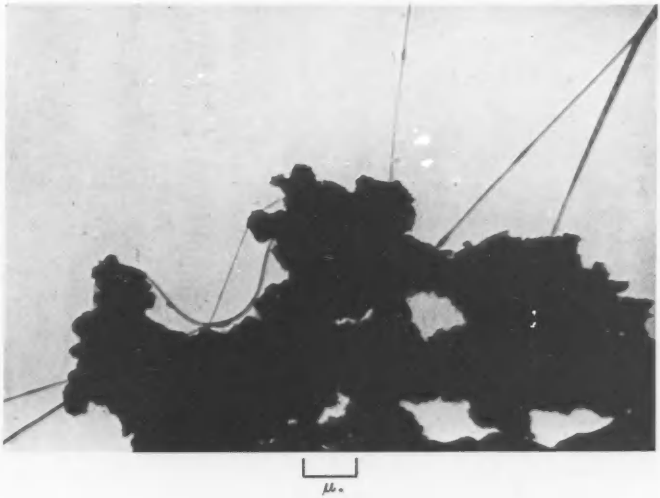

Fig. 10.

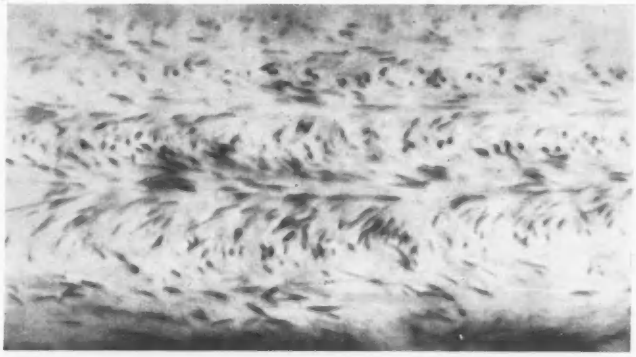

Fig. 11.

grey film (less than 100 A. thick) which apparently is pure cellulose. This is a surface film of the insoluble cellulose on the culture liquid. From this diffuse film the visible threads are crystallized.'

The cells of *A. acetigenum* as depicted on our electron micrographs are considerably larger than the cells of *A. xylinoides*, the former measuring from 43,000 × 10,000 A. to 22,000 × 8000 A., whereas the cells of *A. xylinoides* as shown by Frey-Wyssling & Mühlethaler vary from 20,000 × 5000 A. to 10,000 × 5000 A. Apart from these differences in the sizes of the typical cells of the two species our electron micrographs of preparations from glucose media show cellulose formations having a general resemblance to those depicted in the electron micrographs of Frey-Wyssling & Mühlethaler. The latter state that: 'the equal thread diameter (about 200 A.) in old cellulose films of *A. xylinum* as well as in new films of *Bacterium xylinoides* is rather surprising. Whether this conformity is accidental or whether there is a preferred strand width (which would indicate that the possibility of crystallization is limited) is a problem which should be investigated.'

The uniformity in thread diameter (about 200 A.) reported by Frey-Wyssling & Mühlethaler is not apparent in our preparations, but many of the threads in our own specimens of cellulose from glucose media had a width of approximately 250 A. and, in his latest publication on this topic, Mühlethaler (1949) observes that cellulose fibres produced by *A. xylinum* 'have had from the very first a diameter of about 250 A.'. The 50 A. difference between these two estimated widths (namely, 250 and 200 A.) may perhaps be capable of explanation when account is taken of the fact that, when measuring the threads, the accuracy with which their edges can be defined will depend upon (*a*) the resolving power of the electron microscope used, (*b*) the resolving power of the photographic emulsion used, and (*c*) the conditions of development of the photograph. Our observations lead us to the view that the bacteria produce round their cells mucoid carbohydrate material which is built up to the dimensions of cellulose, whereupon crystallization begins in the manner shown in our figures, as also in those of Frey-Wyssling & Mühlethaler. The latter, in his latest communication, expressed a similar opinion. The electron micrographs of our preparations from media containing glycerol and glycol, respectively, support this opinion, since the amorphous masses shown in these pictures might well consist of lower carbohydrate polymers in process of elaboration to cellulose.

SUMMARY

1. Cellulose membranes produced by *Acetobacter acetigenum* yield an X-ray pattern similar to that revealed by X-ray examination of cotton cellulose.

2. Membranes from very young cultures of *Acetobacter acetigenum* on media containing glucose, glycerol and ethylene glycol, respectively, as sources of carbon, have been examined in the electron microscope.

3. The preparations from the glucose media show cellulose films in which are threads usually of a width either a little less than 250 or approximately 500 A., the latter being in the majority.

4. The preparation from a glycerol medium shows mainly amorphous material with a very few threads or bands of cellulose, of width approximately 500 A. The preparation from the ethylene glycol medium also shows amorphous material with not many fibrils.

5. It is suggested that the amorphous material consists of carbohydrate at a stage of polymerization lower than cellulose.

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EXPLANATION OF PLATES

Figs. 6–10. Electron micrographs of cellulose formed by *A. acetigenum*

PLATE 1

Fig. 1. Cellulose from ethylene glycol medium.

Fig. 2. Cellulose from glycerol medium.

Fig. 3. Cellulose from maltose medium.

Fig. 4. Cotton cellulose.

Fig. 5. Cellulose from glycerol medium. Rotation photograph.

PLATE 2

Fig. 6. Cellulose formed in a glucose yeast-water medium and examined in an unwashed condition. Magnification, ×8400. The majority of the threads are about 500 A. in width.

Fig. 7. Washed material from a glucose yeast-water medium. Magnification, ×8000. Threads mostly about 500 A. wide with a minority measuring a little less than 250 A. Two threads were found to be not greater than 200 A. in width.

Fig. 8. Washed material from a medium consisting of a solution of glucose and inorganic salts. Magnification, ×9000. Most of the threads have a width of approximately 500 A., but one or two threads embedded in film are approximately 250 A. wide.

Fig. 9. Preparation from a medium containing ethylene glycol and inorganic salts. Magnification, ×24,000. This shows what appears to be amorphous matter with a very few fine threads, the narrowest of which has a width of less than 80 A. Others measured less than 200 A. and one band was found to be 250 A. in width.

PLATE 3

Fig. 10. Preparation from a medium containing glycerol and inorganic salts. Magnification, ×8500. Masses of what apparently is amorphous material, together with a very few fine threads, can be seen. The narrowest thread has a width of 250 A., others are 500 A. wide, while yet other bands may be noted varying in width from 750 to 1000 A.

Fig. 11. Sectional photograph of cellulose from a starch hydrolysate medium. Magnification, ×1600.

A Means of Metabolic Investigation of Small Portions of the Central Nervous System in an Active State

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A method is described below by which it is possible for the first time to study the metabolism of small isolated fragments of the central nervous system of higher animals, in a state of electrically induced activity. Increase in the metabolism of slices of cerebral cortex on electrical stimulation *in vitro* was reported recently (McIlwain, 1951*a*). In the apparatus which was used then, direct contact was made throughout the experiment between the slice of tissue and two stimulating electrodes. The tissue weighed about 50–120 mg. It was realized that the scope of such investigations would be greatly increased if they could be applied to smaller fragments of tissue and without fixing them to electrodes. Regional differences in biochemical responses to stimulation, and in the action of drugs on such responses, could then easily be explored. It has now been found possible to study in this way fragments weighing a mg. or less.

EXPERIMENTAL

Guinea pigs and rats of various breeds and ages were used. They were killed by a blow on the neck or by decapitation, the skull opened and the brain removed. Tissue preparations were normally ready for placing in the thermostat (37 or 39°) 15–30 min. after death of the animal. Salines and means of electrical stimulation were those described previously (McIlwain, 1951*a*). A commercial diathermy apparatus was also used. This depended on charging and discharging a condenser from a.c. mains, through a coil and spark gap. A secondary coil, adjustable in relation to the first, gave the impulses which were taken through a high-frequency meter

to the vessels. The discharge consisted of extremely brief spikes (perhaps 0·1 μsec.) of some millions/sec. in frequency.

Electrodes. The problems met in choosing materials for electrodes in the present vessels were similar to those discussed before (McIlwain, 1951*a*). Again, silver was largely used for electrodes which were not required to be sealed through glass. Electrodes which were to be so sealed were made of platinum and, if they were to be in contact with the experimental saline, were plated with gold. Vessels with sealed gilded electrodes were cleaned in the usual way, except that chromic acid mixtures were not used for such vessels on the first few days after the mixtures had been prepared, but only after they had been in use for other cleaning for some days. This was because the impression was gained that traces of free chlorine were present initially but were gradually lost.

The connexions outside the vessel, from electrode to source of current, were made according to Fig. 3*a* of McIlwain (1951*a*) which involved a junction through mercury. It was found that great care was needed when filling and emptying the side arms carrying the mercury, in order to avoid spilling small droplets into the main vessel, as any contact between the mercury and gold immediately resulted in amalgamation. In place of the temporary rubber junctions, more permanent ones were therefore made with a cement or by sealing tungsten wires through the glass.

Design of cylindrical vessels: absorbing carbon dioxide in them. To allow a variety of electrode types to be examined, cylindrical vessels were used with large-sized joints (*C* 24) connecting them to the manometers (Figs. 1 and 2). For the same reason the centre well for gas-absorbing reagents was omitted. These reagents were accommodated in the large

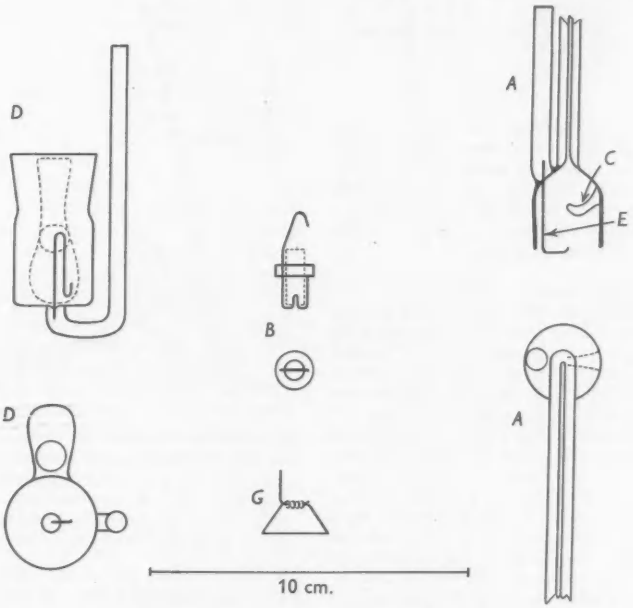

Fig. 1. Cylindrical electrode vessels and accessories. *D*, the vessels, with 1 side arm with valve stopper, and a wire electrode. The electrode would be plated in about the position shown and later bent to a shape such as *I* of Fig. 2, or have attached to it other of the electrodes of Fig. 2. *A*, electrode manometer for vessel *D*; *B*, pendant of silver wire (26 gauge) and polythene for holding a roll of filter paper, shown dotted, for absorbing agents; in use it is attached to the glass hook *C*. *G*, elevation of electrode *G*, Fig. 2, showing how it is attached to the electrode *E* of the manometer.

The platinum wire, which was to form the basis of the electrodes in the conical and cylindrical vessels, was sealed to the vessels in slight excess of the amount estimated to be needed. It was then bent roughly to position and trimmed to a suitable length. It was then plated, and moved at intervals during plating, ensuring especially that no part of the wire was touching the vessel wall except at the point of the seal. It was then bent to its final position with small wooden sticks and with forceps which had small plastic sleeves slipped over them where they were to grip the wire.

head which the manometer carried. Fig. 1*B* shows the pendant devised for this purpose. At the beginning of an experiment a strip of hardened filter paper about 17 × 35 mm. was rolled and put in the polythene ring, so that it rested on the silver bridle. 5 N-NaOH (0·15 ml.) was pipetted as a single drop into a small polythene weighing bottle, the paper in its pendant rested on the drop and the weighing bottle was closed so that atmospheric CO_2 would not be absorbed. This was done 0·5 hr. or more before the absorbent was needed. After saline and tissue had been added to the

manometric vessel and the manometer greased and connected to its oxygen supply, the pendant was taken from its bottle, held below by forceps, its upper loop hung over the glass hook on the manometer head, and the vessel put on the manometer.

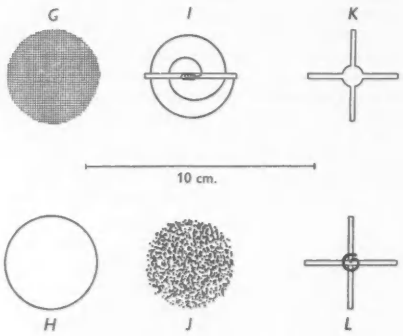

Fig. 2. Electrode patterns referred to in Table 1. *G* (see also Fig. 1) was of silver gauze (wire 0·2 mm. diam., mesh 18/cm.); *H* and *K* of silver sheet; *J* formed on the base of the vessel by a silvering mixture (Johnson, Matthey and Co.); *I* and *L* of gold-plated platinum, if as lower electrodes fixed to the vessel, or of silver wire with the Perspex spacers shown, if hanging.

Fig. 3 gives the result of experiments which were carried out to see how effectively the alkali paper of the pendant absorbed CO_2. It proved to be as effective as the paper in the normal centre wells of conical Warburg vessels of similar volume. The shaking rate in experiments with the cylindrical vessels was about 120/min. This relatively high rate was found necessary in order to keep the tissue suspended.

Effect of passing current in the absence of metabolizing tissue. Various chemical and physical effects of passing currents through saline and substrate mixtures were noted in an earlier paper (McIlwain, 1951*a*). To avoid confusing any of these with a metabolic response of the tissue being investigated, each new electrode arrangement was examined in the following ways. (*a*) For gas change in the saline mixture without substrate; electrolysis by excessive currents or current densities could then be seen. (*b*) For gas change in the presence of substrate and any absorbing agents. This was carried out periodically in all vessels to ensure that the plating was intact. (*c*) For any change in a saline in which tissue had metabolized but had been removed. The effects of a labile metabolic product, not evident in (*b*), would have been seen, but no instance of this was found.

Chopped tissues. These were prepared as follows. The tissue, for example the sheet of cortex obtained by unfolding a guinea pig hemisphere (McIlwain, 1951*a*), was placed horizontally on a pad of several filter papers moistened with the experimental saline, and was first cut in one direction to strips 1–2 mm. wide with razor blading. Keeping the blade vertical, but in a direction approaching right angles to the first direction, the blade was then moved up and down rapidly while the tissue was moved under the blade, in the fashion adopted when chopping vegetables with a knife. The chopped tissue was washed from the filter paper into a small dish of saline with a wide-mouthed Pasteur pipette, and sucked up and down in the pipette a few times in order to separate clumps of fragments which were not completely cut. The tissue was transferred to experimental vessels by scooping it up on a small gauze shovel. This was made from a piece of silver gauze (mesh 18/cm. wire 0·2 mm. diam.) about 8 × 12 mm. mounted in a handle such as those used for bacteriological wire-loops. In this way, extremely small fragments of tissue were allowed to escape through the gauze. The amount scooped up for each vessel was judged

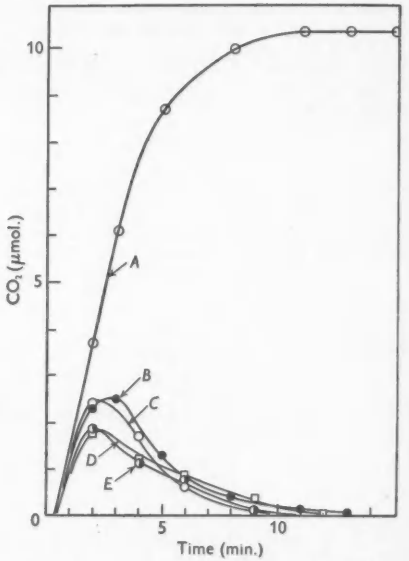

Fig. 3. Showing efficiency of CO_2 absorption by alkali-paper in different vessels. Main compartments of the vessels contained 3 ml. 0·1 N-H_2SO_4 and the side arms, 0·5 ml. 0·02 M-Na_2CO_3. *A*, no absorbing reagents; *B–E*, with filter paper and 5 N-NaOH. *B* and *C*, Warburg vessels of about 18 ml. and *D* and *E*, cylindrical vessels of 23 ml.

visually to be 60–70 mg., when working at 20 mg./ml. and with vessels taking 3–3·5 ml.; after practice this amount could be judged by its appearance on the scoop or in the vessel. At the end of the experiment the contents of each vessel were filtered through asbestos in a Gooch crucible. If needed for analysis, a specimen of the filtrate was then taken and the vessel washed out with 0·9 % NaCl. The crucible, which had previously been prepared by washing, drying and weighing in the same way, was also washed with 0·9 % NaCl, dried at 100° and weighed. Wet weight of cerebral cortex was calculated from the dry weight by the factor 7·1 (McIlwain, 1951*a*).

Homogenates were prepared in all-glass test-tube homogenizers, from 1 part of cerebral cortex and 3 parts of glucose-phosphate saline cooled in ice. The instrument was driven rapidly and homogenizing took 20 sec.; the suspension was passed through a wisp of glass wool in a small funnel and pipetted to the experimental vessels. Another portion was taken for dry weight. For comparison, wet weights were calculated as above.

RESULTS

Stimulation of slices without fixing them to electrodes

Tissue fixed to a variety of electrodes could be stimulated in salines by impulses which were without action on slices floating freely in salines containing similar electrodes (McIlwain, 1951*a*). However, these experiments were arranged so that the free slices did not come between the electrodes, where they would have been in danger of being entangled in them. They were commonly a cm. or so away, and not in the immediate field produced by the applied impulses. Moreover, a stimulus could be effective when only one pole was directly connected to the tissue (Fig. 8 of McIlwain, 1951*a*). A factor conditioning stimulation was likely to be the potential gradient to which the tissue was exposed. Stimulation could be brought about by peak voltages of 3–5 V. between electrodes 2–10 mm. apart. It seemed possible by suitable electrode arrangements to include most of the fluid in an experimental vessel in such a field.

In devising the conditions under which this might be done, vessels *D*, Fig. 1, have largely been used. They and the corresponding manometers, and their handling, are described in the experimental part. The vessels were made cylindrical so that the variety of electrode types of Fig. 2 could be examined. One of these was normally at the bottom of a column of saline about 22 mm. in diameter and 7 mm. deep. The other was parallel to the first and about 5 mm. above, so that it was near the surface of the saline. The tissue was between the two, and by rapid shaking (about 120 complete oscillations of 8 cm./min.) was maintained in suspension in the fluid.

Impulses of characteristics similar to those effective when applied to tissue-holding electrodes were not always effective when applied to the electrodes of vessels *D* (Table 1; Fig. 1). Alternating current was not suitable for stimulation owing to the relatively large amount of energy expended. Thus with electrode *G* above and *H* below, application of 2 V. at 50 cyc./sec. caused a pressure change of some 25 mm. corresponding to a mean temperature increase of about 0·8°. No increase in respiration resulted (Fig. 4*B*). Use of electrodes *J* or *K* below, retaining *G* above, was no more satisfactory. In attempts to apply greater potential gradients across the vessel condenser pulses were used, as these could give a much higher peak voltage with a given amount of energy. Pulses at 24 V. and reasonable capacities and frequencies were, however, again unsuccessful with electrodes *G* and *H*. Thus from a 4 μF. condenser charged and discharged 50 times/sec. (charging potential 24 V.) through the vessel, a mean temperature rise of about 0·25° was caused without any

Table 1. *Effects of applying currents to slices in cylindrical vessels with various electrodes*

(Experiments employed manometers and vessels *D*, Fig. 1, with the electrodes *G–L* of Fig. 2. Slices of about 60 mg. from guinea pig cerebral cortex were floated freely in 3–4 ml. of oxygenated glucose phosphate saline, between the electrodes. Experiments were normally arranged with an initial unstimulated period of 30 min., followed by one or more periods during which impulses were applied. After or between periods of stimulation, the unstimulated rate was again determined. With condenser pulses (*C*) the maximum increase in temperature was 0·2°; with alternating current (a.c.) the increases were 0·2 and 0·8° respectively. The rate of formation of lactic acid during stimulation was calculated by supposing that it had been regular, and that the increase observed in the stimulated vessel had all taken place during the stimulation. Evidence for this has been given (McIlwain, Anguiano & Cheshire, 1951).)

Electrodes		Impulses			Respiration			Lactic acid formation		
						Stimulated		Unstimulated	Stimulated	
Upper	Lower	Type	V.	Duration (msec. to fall to ½ peak value)	Unstimulated (µmol./g./hr.)	(µmol./g./hr.)	% of unstimulated	(µmol./g./exp.)	(µmol./g./exp.)	Rate (% of unstimulated)
G	*H*	a.c.	1·25	(138 mA.)	70	68	97	—	—	—
G	*H*	a.c.	2·0	(286 mA.)	70, 65	66	98	—	—	—
G	*H*	*C*, 4 µF.	20	—	65	68	104	—	—	—
K	*I*	*C*, 4 µF.	24	—	65, 63	63	98	—	—	—
L	*I*	*C*, 4 µF.	24	0·8	63	98	155	43	83	315
I	*L*	*C*, 1 µF.	48	0·2	63, 63	92	145			
I	*L*	*C*, 4 µF.	24	0·8	62	87	140			
I	*L*	*C*, 4 µF.	48	0·8	62	128	207	45	98	363
I	*L*	*C*, 4 µF.	12	0·8	62, 58	72	120			

stimulation. However, the resistance of the vessel was so low that the discharge time was very brief. It was not measured accurately, but from the characteristics quoted in Table 1 it can be calculated that the time taken for the applied potential to fall to one-third of its peak value was about 30 μsec. This is likely to be much shorter than the time needed for stimulation at minimum voltage (for references see McIlwain, 1951*a*).

These observations showed that it was desirable to increase the resistance of the experimental vessel; this would decrease the energy expended by an alternating current, and increase the duration of condenser pulses. The simplest means of increasing resistance was to decrease the area of the stimulating electrodes. Electrodes I to L of Fig. 2 were made with this in view. Table 1 shows that the combination K and I was still ineffective. On the other hand,

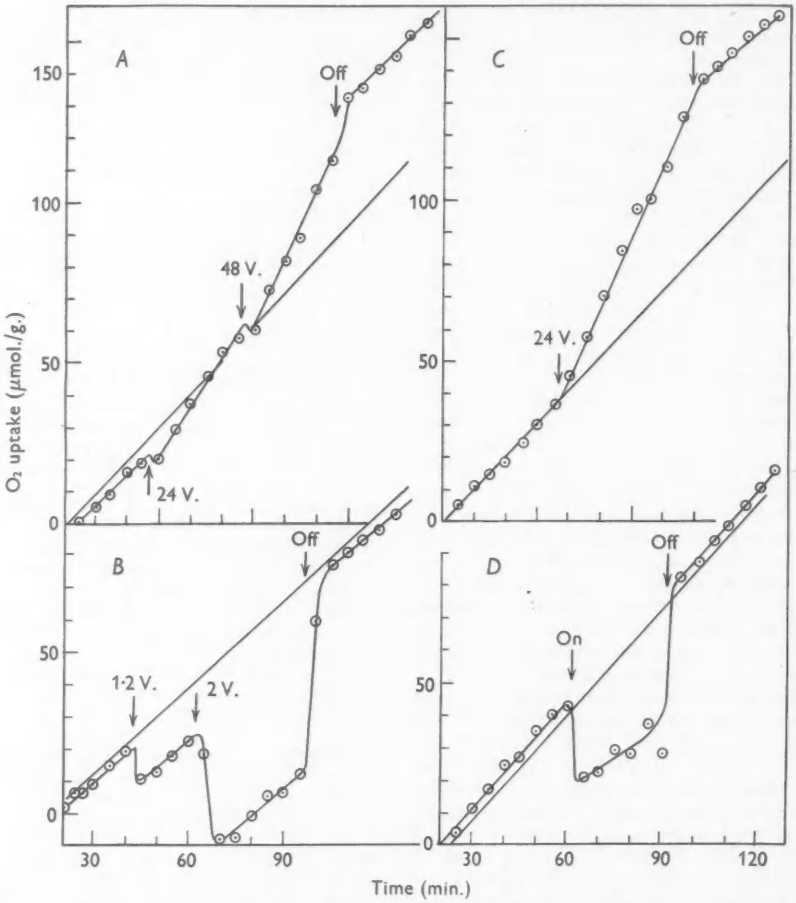

Fig. 4. Effective and ineffective impulses applied to various preparations of cerebral cortex. Experimental points are not indicated for the unstimulated control vessels. *A*, slice in cylindrical vessel, stimulated by condenser (4 μF.) pulses, 100/sec. at peak voltages quoted. *B*, slice in cylindrical vessel with electrodes *G* and *H*. Alternating current, applied as indicated, had a large heating effect, but caused no change in respiratory rate. *C*, Slice fragments in conical vessel, stimulated as *A*. *D*, Slice fragments of about 3 mg. in a cylindrical vessel; current applied from a diathermy apparatus during the period indicated.

a simple wire end, rounded and some 1 mm. across, was too small: evolution of gas took place from it when pulses at 24 V. from a 4 μF. condenser were applied. This did not occur when the condenser was reduced to 0·5 μF., but the pulses were then too brief.

The best compromise appeared to lie in relatively short lengths of wire, and the combination of I and L was successful. Table 1 shows that with these electrodes, large increases could be obtained in both the respiration and glycolysis of slices. The increases could be fully equal to those obtained by electrodes which made contact with the slices and also by other means (see McIlwain, 1951*a*). Again, the proportional increase in glycolysis was greater than that in respiration. Threshold values, and the type of pulse most effective under the two conditions, have not been compared in detail. It is evident from Fig. 4, however, that response depends on the character of the impulses applied and is independent of their heating effects; a.c. currents were presumably ineffective because of the relatively small potential gradient or current density, and the high frequency pulses because they were too brief to excite.

Stimulation of fragments of tissue

When stimulation had been obtained without attachment of tissue to electrodes, it became possible to investigate fragments of tissue, which could be handled as suspensions. To find whether the response depended on the size of tissue-fragment, slices, prepared in the ordinary way and known to be capable of response, were cut with sharp scissors to many smaller fragments. After cutting to pieces 1–5 mg. in weight, their respiration and glycolysis remained at values very close to those in slices of 60–120 mg. Thus, the unstimulated values during the experiments of Table 2 were 65–73 μmol. O_2/g. wet wt./hr. and 18–25 μmol. lactic acid/g./hr.

Pulses effective with slices were effective with fragments of slices. Table 2 quotes instances of increase in respiration to 170 % and of glycolysis to 300 % of the unstimulated values. Fragments of the tissue occasionally became caught and attached to one of the stimulating electrodes, but for most of the time all were suspended and no marked difference was seen to result from the occasional attachment. Further, response could be obtained in the much finer suspensions of tissue obtained by chopping the whole cerebral cortex. The making and handling of such preparations is described in the experimental part.

In the instances tried, little or no response has been given by homogenates (see Table 2). The homogenates were made (see above) and studied in the same saline as that used for the slices. This may not

Table 2. *Response to applied currents by fragments of guinea pig cerebral cortex*

(Cylindrical vessels were D of Fig. 1, with a spiral I (Fig. 2) below and L above. Glucose-phosphate saline was used with O_2; NaOH paper above. Stimulation was by diphasic condenser (4 μF.) pulses at 100/sec. applied from a battery of 24 V.; when measured from the vessel terminals by an oscilloscope they were found to be 20–23 V. and 0·5–0·8 msec. was required for fall to one-third of the peak voltage. The rate of lactic acid formation was calculated as in Table 1.)

Tissue preparation (see text)				Respiration			Lactic acid formation			
				Unstimulated	Stimulated		Unstimulated	Stimulated		
Vessel	Type	Average wt. of fragments (mg.)	Impulses	(μmol./g./hr.)	(μmol./g./hr.)	% of unstimulated	(μmol./g./exp.)	(μmol./g./exp.)	Rate (% of unstimulated)
Cylindrical	Sliced, cut	3·2	Condenser	63	90	145	34	44	205
Cylindrical	Chopped	0·3	Condenser	57	85	142	44	71	264
Cylindrical	Sliced, cut	3	Diathermy (see experimental section)	70	Approx. 70	Approx. 100	84	89	112
Cylindrical	Homogenized	—	Condenser	45, 40	43	100	28	25	90
Conical	Sliced, cut	5·6	Condenser	75, 71	174	234	34	58	315
Conical	Sliced, cut	3·2	Condenser	65	150	233	41	81	280
Conical	Chopped	0·3	Condenser	68, 64	109	164	29	45	310
Conical	Homogenized	—	Condenser	32	36	112	21	23	109

be an optimal one (see however, Elliott & Libet, 1942; Elliott, 1948; and Cavanaugh, 1950), but we have not at present explored preparations intermediate between this and the chopped brain.

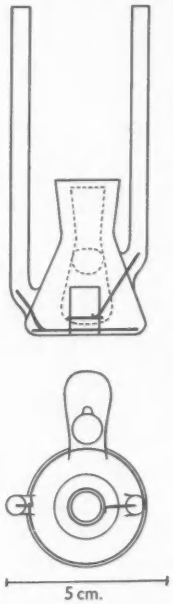

Fig. 5. Conical electrode vessels *E*. The heavier lines represent the wire electrodes.

Conical vessels

The cylindrical vessels were initially devised with the intention of placing tissue within an electric field which would be uniform at any one time. Other factors led to departure from this intention, and the most successful combination of electrodes, reached as a result of the experiments described above, had its greatest field density within a shape which approached a cone. Conical vessels have many advantages in manometric work. Agitation of fluid and surface-volume relationships are more satisfactory than in cylindrical vessels. Various electrodes have therefore been placed within ordinary conical Warburg vessels. The arrangement finally chosen was the simple one shown in Fig. 5. Here one wire is placed round the outside of the centre well towards the top of the expected fluid level, and another round the inside angle of the base. In developing this, the size of the vessel was chosen as being one commonly used. Different gauges and lengths of wire were then tried in order to arrive at those which, with a moderate-sized condenser, gave vessels of such a resistance that the time of discharge would approach that known to give a good metabolic response. The size of condenser was chosen as being one which did not give too great a heating effect when charged and discharged through the saline at some 50 times/sec. (for choice of this frequency see McIlwain, 1951*a*). A single turn of each wire was found preferable as giving least opportunity for entangling tissues. The sizes of wire found suitable were between 0·19 and 0·46 mm. in diameter (36 and 26 gauge). The plating brought these sizes to about 0·21 and 0·48 mm.

Excellent metabolic responses were obtained in these vessels with fragments of tissue (Table 2). Both respiration and glycolysis increased greatly in slices and in chopped brain cortex.

Application to various parts of the brain, and to the action of drugs

Preliminary indications are given in Table 3 of the application of the present methods. The cerebellum of many small mammals is convoluted so that if slices are cut from it in the ordinary way, fragments of a few mg. in weight are obtained. These can, however, be handled satisfactorily by present methods, and were found to respond to stimulation. Adequate preparations were also obtained from basal ganglia and from the brain stem.

Table 3. *Response to applied currents by different parts of the brain; action of atropine*

(Experiments employed conical vessels and diphasic condenser pulses of a peak potential of 20–23 V. and duration (at one-third max. voltage) of 0·5–0·8 msec. The tissue was as fragments of 1–4 mg. in 3·5 ml. glucose-phosphate saline with Ca salts. The successive periods were each of about 30 min., beginning 20–30 min. after death of the animal; after the second, atropine was tipped in from the side arm of the vessel.)

	Respiration in successive periods (μmol./g. dry wt./hr.)			
	Before addition		With 10^{-3} M-atropine	
Tissue	Unstimulated	Stimulated	Stimulated	Unstimulated
Guinea pig cerebral cortex	530	1230	805	505
Guinea pig cerebellar cortex	570	1140	810	535
Guinea pig basal ganglia	400	710	—	—
Rat cerebellar cortex	620	1320	730	590
Rat brain stem	230	360	260	210

Substances can be added to the metabolizing tissue from the side arms of the vessels without interrupting the electrical stimulation. A survey of various agents showed that some, including atropine, greatly decreased the electrically stimulated respiration and glycolysis of slices fixed to electrodes; they were, however, without action on the same processes in unstimulated slices (McIlwain, 1951*b*). Table 3 shows that the respiration and glycolysis of fragments of brain, unattached to electrodes, were also unaffected by atropine in the unstimulated condition, but during electrical stimulation the processes were considerably inhibited by atropine. The activity induced in the fragments is likely to be different in certain respects from that in the intact brain, but the present observations together with those previously described (McIlwain, Anguiano & Cheshire, 1951; McIlwain, 1951*b*) indicate many similarities.

DISCUSSION

The only methods previously available for studying the metabolism of the central nervous system in different states of activity have been those employing whole animals. Perfusion has been little used. The main methods have been (*a*) analysis of blood entering and leaving the organ, with measurement of its rate of flow; and (*b*) analysis of the brain itself, often after rapid fixing and dissection. Isotopic and potentiometric techniques (Davis, McCulloch & Roseman, 1943–4; Gurdjian, Webster & Stone, 1946) can be regarded as special instances of these general methods.

The present procedure may be described as an *in vitro* method in distinction to the previously available *in vivo* ones. As such it is complementary to them. It would have an important analytical function even if the other methods were easy to apply. This, however, is not the case. Thus, many of the changes which accompany different states of activity in the brain are in rates rather than in types of metabolism. The *in vivo* methods give metabolic rates only indirectly and after many individual analyses. With the method of fixing and analysis, the analyses are necessarily on different groups of animals fixed at different times in relation to the experimental treatment. In the case of determination by arterial-venous difference, measurement of blood flow itself may need many individual analyses (Schmidt, 1950). This contrasts sharply with the present method in which direct measurements are obtained of oxygen uptake or acid formation in the same piece of tissue under different conditions, and in which very different conditions can, if necessary, be applied to pieces of material from adjacent parts of the brain.

Further, it is not easy to apply the *in vivo* methods, including perfusion, to small parts of the brain. To do this by arterial-venous difference would mean tapping the blood entering and leaving the particular area, and this can be done only to a very limited extent. Fixing and analysis can be applied to parts of the brain when these are readily recognizable, and when substances which are stable in the tissue are involved, but are not applicable to processes such as respiration. Also with, for example, phosphate fractions the method would require freezing and dissecting the frozen tissue in which it is especially difficult to separate the same small area from each of a series of animals.

Considering the analytical function of the present method, it is noteworthy that much of the present knowledge of whether a substrate will support the activity of the brain is derived with difficulty from experiments *in vivo* with hypoglycaemic animals. Agents, normal to the body or not, can be applied to the tissues *in vitro* with greater certainty that the agents themselves will be responsible for any observed change. The separated tissue is obviously physically altered in preparation and in an unusual environment, but many of its constituent elements need not be and can be examined under a range of electrical and chemical conditions much wider and more closely controlled than those which can be applied in a whole animal.

The present techniques can obviously be applied to tissues other than those of the brain. This study has been commenced in these laboratories (Kratzing, 1951). Smaller apparatus has also been made on the present principles, and in instances when manometric measurements are not required, very simple arrangements of two wires in a vessel would be adequate for stimulation. We have also, in working with the brain, reported largely investigations of energy-yielding and associated processes, although the present methods are applicable also to studying changes more immediately associated with the passage of nerve impulses.

The methods now developed for stimulating tissue-fragments without fixing to electrodes do not entirely replace those in which slices are held in stimulating electrodes. When sufficient coherent material is available, stimulation by contact with relatively large pieces of tissue remains most suitable for studying labile tissue constituents, for the application of defined potential gradients especially in particular directions in relation to the tissue, and for the application of certain types of impulses.

SUMMARY

1. Apparatus has been devised so that fluctuating potential gradients can be applied to portions of tissues of between 0·2 and 100 mg. in weight, while these are floating freely in oxygenated nutrient salines.

2. Condenser pulses, of time constant about 0·5 msec., could be applied in this apparatus from condensers of about 4 μF. without causing any changes which might be mistaken for metabolic ones. A maximum potential gradient of about 20 V./cm. was found to alter the metabolism of pieces of cerebral cortex which were floating in the apparatus in a balanced glucose saline. Respiration increased to up to 200 % of its initial rate and lactic acid formation to some 300 %.

3. Similar increases were shown with cerebellar tissues and fragments from the basal ganglia and brain stem. The increases were partly inhibited by concentrations of atropine which had very little, if any, effect on the levels of respiration and glycolysis normal to the tissues.

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Further Investigations on Fish Tropomyosin and Fish Nucleotropomyosin

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The first method for preparing tropomyosin from muscles of several animals (rabbit, pig, horse and whiting) was described in 1948 by Bailey. Minced muscle is dehydrated with organic solvents and extracted with M-potassium chloride for 12 hr. Tropomyosin is isolated from the extract by precipitating at pH 4·3–4·5, redissolving in water at neutral pH, and fractionating with ammonium sulphate between 45 and 70 %. The amount of tropomyosin in rabbit muscle has been estimated by this method to be 0·47 g./100 g. fresh muscle weight.

Another method of preparation has been given recently by the author for carp muscle (Hamoir, 1951). The minced muscle is submitted to two successive extractions for short periods of time (10–20 min.) with potassium chloride-phosphate solutions: the first of pH 5·0 ($I = 0{\cdot}25$), the second of pH 5·5 ($I = 0{\cdot}6$) containing 0·3 % sodium adenosinetriphosphate. The first extract contains the muscle globulins soluble at low ionic strength and some tropomyosin; the second myosin, nucleotropomyosin and tropomyosin. The method of isolation of nucleotropomyosin and tropomyosin from these extracts does not differ essentially from Bailey's method. Nucleotropomyosin can easily be separated from tropomyosin: it coprecipitates with the myosin at low ionic strength and neutral pH while tropomyosin remains in solution. The total yield of tropomyosin and nucleotropomyosin isolated by this method from fish muscle does not amount to more than 0·1 g./100 g. fresh muscle weight.

The difference in yield by the two methods has led us to try to account for the discrepancy. Fish muscle was treated following the author's method and tropomyosin was extracted from the residue by Bailey's (1948) method. The total yield of tropomyosin was then comparable with that obtained by Bailey for rabbit muscle. New investigations have also been carried out on fish nucleotropomyosin. The content of ribonucleic acid and its splitting under various conditions have been examined more closely, and the ribonucleic acid has been more fully characterized by its ultraviolet absorption in N-perchloric acid and by quantitative estimation of the nitrogenous bases present.

METHODS

Preparation of fish tropomyosin and fish nucleotropomyosin

Carp muscle was used as starting material. Tropomyosin was prepared from the residue obtained after extracting twice by the author's method (Hamoir, 1951) and subsequently treating it according to Bailey's (1948) method. As some

simplifications have been introduced, a description of the preparation will be given here.

The residue was washed with 2 vol. of water (200 ml./100 g. fresh muscle) and 1 vol. of 1:1 ethanol-water. The dehydration was continued through two changes of ethanol (1 vol.) and two of ether (1 vol.). The pulp was immersed while still ether-damp in 7 vol. of M-KCl (700 ml./100 g. pulp) at pH 7 and was extracted with stirring for 12 hr. at room temperature. The insoluble residue was removed by centrifugation (10 min. at 10,000 rev./min.) and re-extracted twice for 2 hr. at neutral pH. Tropomyosin was precipitated from the combined extracts by first diluting with 9 vol. of water and acidifying to pH 4·6. The precipitate was washed twice with a dilute solution of acetate buffer (pH 4·6), and redissolved in 0·5 M-KCl at neutral pH. The solution was then fractionated with neutral $(NH_4)_2SO_4$. The fraction isolated between 50 and 66% saturation corresponds to practically pure tropomyosin. The purity was assessed by electrophoresis (Dubuisson, Distèche & Debot, 1950) and by ultracentrifugation at 60,000 rev./min. (Spinco electric-driven ultracentrifuge). The isolation and purification of nucleotropomyosin were carried out as previously described (Hamoir, 1951).

The solutions of tropomyosin and nucleotropomyosin were usually dialysed for at least 1 week against several changes of NaCl-acetate buffer ($I = 0{\cdot}4$ and pH 6·4) to remove any phosphate or ammonium ions present.

Analytical

Protein. Total nitrogen was estimated by the micro-Kjeldahl method and the concentrations of tropomyosin and nucleotropomyosin were calculated by assuming a N content of 16·7%, given by Bailey (1948) for tropomyosin. No appreciable error was introduced in this way in the evaluation of nucleotropomyosin, the nitrogen content of nucleic acid being close to that of tropomyosin (Smith & Markham, 1950).

Phosphorus. Total phosphorus was estimated by the perchloric acid method as modified by Allen (1940), using a photoelectric absorptiometer and the 608 Ilford filter.

Phospholipins. The ethanol- and ethanol-ether-soluble compounds of nucleotropomyosin were removed following the method of Ogur & Rosen (1950). The protein was precipitated with 5 vol. of ethanol and extracted with two changes of ethanol at room temperature. The residue was resuspended in a mixture of 3:1 ethanol-ether and boiled gently on a water bath for 5 min. The process was repeated and the extracts combined. After the removal of solvent, the amount of phospholipin was determined from the phosphorus content.

Ribonucleic acid. The absence of ribonucleic compounds in the phospholipin fraction was checked by showing the absence of ribose. Ribose was determined by the method of Mejbaum (1939), adopting a 45 min. heating time for the development of the colour as proposed by Albaum & Umbreit (1947). The readings taken with a photoelectric absorptiometer and the 607 Ilford filter were referred to a calibration curve determined with D-ribose. The nucleotropomyosin precipitated by the organic solvents was dried *in vacuo* and used for the determination of the ultraviolet absorption of the nucleic acid and for the chromatographic analysis of the purine and pyrimidine bases. For the former, the residue was extracted with N-$HClO_4$ at room temperature for about 24 hr. according to Ogur & Rosen (1950) and the absorption of the extracted ribonucleic acid was measured with a Beckman universal spectrophotometer (model DU). The data were calculated as extinction/gram atom of phosphorus. For chromatographic analysis, the paper chromatographic method of Smith & Markham (1950) was slightly modified. Hydrolysis was carried out in N-HCl at 100° in a water bath for 20 min. instead of 1 hr. The extraction of the bases is complete under these conditions and the protein does not dissolve appreciably (Thomas, 1951). After elution of the spots of the chromatogram, the densities of the solutions were measured with the Beckman spectrophotometer. Concentrations were evaluated using the coefficients given by Smith & Markham (1950).

RESULTS

Tropomyosin content of fish muscle

Tropomyosin was estimated by Bailey's (1948) method on the residue left after dissolving out nucleotropomyosin and some tropomyosin following Hamoir's (1951) method. The extraction and isolation were carried out as quantitatively as possible. The yield of tropomyosin calculated from the nitrogen content of the solutions obtained amounted to 0·3% of the fresh muscle weight. As about 0·1% tropomyosin was removed before the treatment with organic solvents, the total content is approximately 0·4% of the fresh muscle weight.

This estimate is based on the supposition that the product is pure tropomyosin. As the protein was prepared by Bailey's method, the presence of significant amounts of nucleic acid seems unlikely. This was confirmed by determining the P content of two preparations, and the values of 0·05 and 0·13% found show that variations occur from one preparation to the other; however, the contamination with nucleic compounds is not very great. Electrophoresis and ultracentrifugation show that tropomyosin proper constitutes at least 95% of the total protein material. A small peak was found by electrophoresis migrating with a mobility of about 0·4 times that of tropomyosin. In the ultracentrifuge, this material sedimented with a rate 1·1 times that of tropomyosin. It is perhaps worth while to mention that this impurity seems not to be present in preparations in which the extraction of the dried residue was not exhaustive: a single extraction with M-potassium chloride or the extraction of a dried residue kept for a few weeks at room temperature gave lower yields of a tropomyosin which was pure by electrophoresis and ultracentrifugation.

In conclusion, the major part of tropomyosin of fish muscle does not go into solution by salt extractions of short duration at pH 5–6 and can easily be isolated from the residue after dehydration with organic solvents. Although the tropomyosin content of fish muscle has been assessed by the use of three types of extraction used in succession, the accuracy is sufficient to show that the amounts in carp and rabbit muscles are comparable.

The phosphorus of nucleotropomyosin

Since ribonucleoproteins are usually associated with phospholipins in the cytoplasm of animal cells, and since variations in the phosphorus content of nucleotropomyosin were observed, the nature of the phosphorus compounds of several preparations has been investigated. Nucleotropomyosin does not contain phospholipins. If the nucleoprotein is extracted with ethanol at room temperature or with boiling mixtures of ethanol and ethyl ether, no phosphorus or ribose is found in the extracts after evaporation of the organic solvents. The variations of the phosphorus content of several preparations are thus due to changes in the proportions of tropomyosin and nucleic acid. Table 1 shows that the content in nucleic acid can vary from 15 to 20%.

Table 1. *Phosphorus content of nucleotropomyosin*

Preparation	P content (% of dry wt.)	Amount of nucleic acid* (%)	P (atom/mol. tropomyosin)†
A	1·20	14·3	24
B	1·26	15·0	25
C	1·72	20·5	37
D	1·48	17·6	31
E‡	1·47	17·5	30
Mean value	**1·43**	**16·98**	**29**

* Assuming a P content of 8·4% of yeast nucleic acid (Smith & Markham, 1950).

† Assuming a molecular weight of 53,000 for tropomyosin (Tsao, Bailey & Adair, 1951).

‡ Taken from the previous paper (Hamoir, 1951).

Splitting of nucleotropomyosin at pH 3·5

It has been shown that nucleotropomyosin kept in the cold in an acetate buffer of $I = 0{\cdot}15$ and pH 3·5 (acetate, 0·1 M and sodium chloride, 0·05 M) transforms slowly: a precipitate forms which is easily removed by centrifugation, and ultracentrifugation of the supernatant showed a progressive disappearance of nucleotropomyosin and the formation of tropomyosin (Hamoir, 1951). It was sought to discover whether this splitting is due to a degradation of the nucleic acid or corresponds to a separation of the nucleic acid and the protein. Dilute solutions of nucleotropomyosin kept in the cold were dialysed overnight in cellophan bags against water and afterwards against a small volume of the acetate buffer of pH 3·5 for 2 or 3 days in order to obtain a complete disappearance of nucleotropomyosin in the supernatant. No nucleic compounds were found in the diffusate: tests for ribose, phosphorus and nitrogenous bases (ultraviolet absorption) were negative. By centrifuging of the contents of the bag, a supernatant was obtained which contained tropomyosin and only small amounts of phosphorus (Table 2). The nucleic acid thus appeared to be bound to the insoluble fraction. This was washed twice with acetate buffer of pH 3·5, resuspended in a solution of $I = 0{\cdot}4$ and pH 6·4 (acetate buffer, 0·1 M, and sodium chloride, 0·3 M) and dialysed for a few days against the latter buffer; a partial dissolution was observed. The phosphorus and the nitrogen contents of the redissolved fraction are given in Table 2, together with values determined on the fraction soluble at pH 3·5.

These results show that when nucleotropomyosin is kept for 2 or 3 days at pH 3·5, it splits into tropomyosin and an insoluble fraction containing practically all the nucleic acid, and from which nucleotropomyosin of a nucleic acid content of about 40% can be prepared. This is insoluble at low pH and differs, therefore, in solubility from tropomyosin itself. When the duration of the acid treatment of the original nucleotropomyosin amounted to 2 or 3 hr., the ultracentrifuge has revealed components sedimenting at a rate intermediate between those of tropomyosin and of nucleotropomyosin (Hamoir, 1951). It can therefore be concluded that 'artificial' nucleotropomyosins can be obtained of a nucleic acid content lower than 15% or higher than 20%, and that it seems probable that the proportions of the two components can be altered continuously.

Ultraviolet absorption of the nucleic acid

The ultraviolet spectrum of the nucleic acid in N-perchloric acid is given in Fig. 1. The curve obtained conforms with the previous values reported on nucleotropomyosin (Hamoir, 1951), taking into account the fact that the extraction with perchloric acid slightly affects the absorption (Ogur & Rosen, 1950). A small shift of the maximum from 258 to 261 mμ. occurs while a trough corresponding to 231 mμ. is now accurately defined. The noticeable absorption at about 280 mμ. already observed with nucleotropomyosin is due to the high content of the nucleic acid in cytosine and guanine (see below). The ϵ_p, i.e. the extinction of a solution

Table 2. *Composition of two fractions resulting from the splitting of nucleotropomyosin at* pH 3·5

Preparation	Fraction considered	P content (% of dry wt.)	Amount of nucleic acid* (%)	P (atom/mol. tropomyosin)*
A	Supernatant	0·38	4·5	7
	Precipitate redissolved	3·28	39·0	92
B	Supernatant	0·19	2·3	3
	Precipitate redissolved	3·3	39·3	93

* Calculated as in Table 1.

containing one gram atom of phosphorus/l. measured in a cell 1 cm. thick, has been calculated at 261 mμ. for several preparations: values of 10,420, 10,900 and 10,460 were obtained, giving a mean of 10,590. Only slightly different (10,800) is the value found by Ogur & Rosen (1950) for yeast ribonucleic acid after perchloric acid treatment.

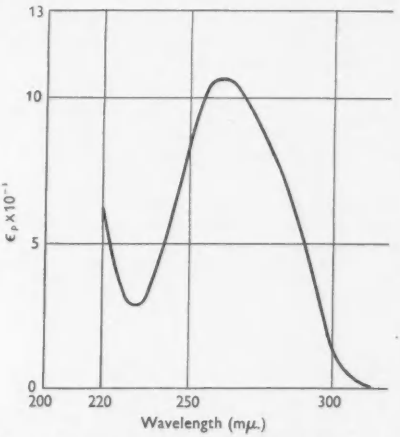

Fig. 1. Ultraviolet absorption spectrum of the ribonucleic acid of fish nucleotropomyosin in N-perchloric acid. Ordinate: extinction of a solution containing one atom P/l. in a cell 1 cm. thick. Abscissa: wavelength (mμ.).

Chromatographic analysis of the nitrogenous bases

A better characterization of the ribonucleic acid of nucleotropomyosin has been gained by the quantitative analysis of the purine and pyrimidine bases present. Four paper chromatograms were run with four different preparations, giving in each case four well separated spots; these were eluted and each compound characterized by its ultraviolet spectrum. The spots correspond to guanine, adenine, cytidylic and uridylic acids. The relative concentrations found are given in Table 3, the adenine concentration being taken as unity. The proportions of the different bases differ notably from the values obtained for yeast nucleic acid (Smith & Markham, 1950), and such high proportions of guanine and cytosine have already been found in the ribonucleic acids extracted from pig pancreas (Vischer & Chargaff, 1948) and from ox pancreas (Kerr, Seraidarian & Wargon, 1949) and seem to be characteristic of animal ribonucleic acids. As deoxyribonucleic acid is only slightly hydrolysed in these conditions and does not move on the chromatogram (Thomas, 1950), the lack of absorbing material at the starting point affords an independent confirmation of the absence of deoxyribose in nucleotropomyosin.

DISCUSSION

A more accurate representation of nucleotropomyosin emerges from the present results. The compound is not a molecular entity but an association of ribonucleic acid and tropomyosin in varying proportions. Some variations occur from one preparation to another and the amount of nucleic acid can be greatly increased by keeping nucleotropomyosin at pH 3·5, when a part dissociates leaving a fraction richer in nucleic acid.

Nucleotropomyosins of a nucleic acid content of 15–20 % have the same solubility properties as tropomyosin: they are salted out by ammonium sulphate in the same range of concentration; they precipitate at low ionic strength at the same pH and crystallize under identical conditions. When, however, the nucleic acid content reaches about 40 %, a difference in solubility is observed at low pH.

Nucleotropomyosin probably pre-exists in muscle and seems not to be formed during extraction. Ribonucleic acids occur always as ribonucleoproteins in the cells (Szafarz, 1951), and there is no reason at present to suppose a splitting of the original ribonucleoprotein and a subsequent combination of tropomyosin and nucleic acid.

To determine the tropomyosin content of fish muscle, three extractants have been used successively. Three tropomyosin fractions were thus obtained which showed surprising variations in composition: the first and the third contained only small amounts of phosphorus while the second was mainly nucleotropomyosin. If nucleotropomyosin pre-exists in muscle, and if no specific variations have to be considered between fish and mammals, the very low phosphorus content of tropomyosin found by Bailey (1948) could be attributed to a splitting of the nucleoprotein in the fibre in the course of dehydration with organic solvents. The most likely although very hypothetical explanation of the previous results seems, therefore, to be that tropomyosin may exist in muscle partly dissolved in

Table 3. *Mole ratio of bases in the nucleic acid of nucleotropomyosin*

	Preparation 1	Preparation 2	Preparation 3	Preparation 4	Mean value
Adenine	1	1	1	1	1
Guanine	2·0	2·1	2·0	2·3	2·1
Cytosine	1·8	1·9	1·8	1·9	1·85
Uracil	1·0	1·15	(0·6)*	—	1·1

* Minimal value due to a too high blank; not used for the calculation of the mean.

the muscle juice in minute quantities (first fraction) and as nucleotropomyosin very partially extracted in the conditions used (second fraction) and whose major part is split into tropomyosin and nucleic acid by dehydration with organic solvents (third fraction). The content in ribonucleic acid of the fibre of skeletal muscle which amounts in the case of the rat to 0·06–0·12 % (Schneider & Klug, 1946) agrees with this hypothesis. The question arises whether the strong basophily of the anisotropic bands of striated muscle (Dempsey, Wislocki & Singer, 1946; Clavert, Mandel & Jacob, 1949) could not be due to the location of nucleotropomyosin at that level in the cell, but more experiments are obviously needed to determine how and where tropomyosin occurs in the muscle fibre.

SUMMARY

1. Fish muscle contains approximately 0·4 % of tropomyosin and does not differ noticeably, therefore, from rabbit muscle in this respect.

2. Nucleotropomyosin does not contain phospholipins; its content of nucleic acid varies between 15 and 20 % and can be increased to 39 % by a partial splitting at pH 3·5.

3. The ultraviolet spectrum and the extinction coefficient of the ribonucleic acid of nucleotropomyosin in perchloric acid do not differ much from the same values of yeast nucleic acid.

4. The ribonucleic acid of nucleotropomyosin contains roughly twice as much guanine or cytosine as adenine or uracil, a composition which appears to be typical of animal ribonucleic acids.

5. It is suggested that, *in situ*, nearly all the muscle tropomyosin could be associated with ribonucleic acid and be located in the anisotropic bands.

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